DOCTOR OF PHILOSOPHY

The role of VEGF-induced PI3K/Akt signalling pathway in head and neck cancer cell migration

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THE ROLE OF VEGF-INDUCED PI3K/AKT SIGNALLING PATHWAY IN HEAD AND NECK CANCER CELL MIGRATION

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THE ROLE OF VEGF-INDUCED PI3K/AKT SIGNALLING PATHWAY IN HEAD AND NECK CANCER CELL MIGRATION

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Thesis submitted for the degree of Doctor of Philosophy

in Cell and Molecular Biology

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DEDICATION

This thesis is dedicated to my sweet and loving mother, Ayesha Khatun and father, Mohammad Abdur Rahim whose love, encouragement, affection and prayers make me able to achieve such success and honour. To my siblings, for their huge support and inspiration. To my wife, Jarin Sultana for her endless love, patience and for being there in times of need. Finally, to my one and only daughter, Jaiyana Islam who brings a new light in my life.

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I am extremely grateful and indebted to my parents, wife, siblings and all my relatives for their increasing encouragement and support.

I would also like to acknowledge the Scottish Overseas Research Student Award Scheme, Dean, Dundee Dental School and Anonymous trust to support me and this project financially.

DECLARATION

I declare that I am the author of this thesis and that I have consulted all the references cited. The work of which this thesis is a record has been accepted for a higher degree. This work has been carried out in the Cell and Molecular Biology laboratories of Dundee Dental School, under the supervision of Dr. Ian R Ellis and Dr. Sarah J Jones.

Signature …….

Date ……

Mohammad R Islam

CERTIFICATE

I hereby certify that Mohammad R Islam has fulfilled the condition of Ordinance 39 of the University of Dundee and is qualified to submit this thesis for the degree of doctor of philosophy.

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ABBREVIATIONS

AICR- American Institute for Cancer Research

AIDS- Acquired Immune Deficiency Syndrome

BSA- Bovine Serum Albumin

CAF- Cancer-associated Fibroblast

cAMP- Cyclic Adenosine Mono Phosphate

CSC- Cancer Stem Cell

DAB- 3, 3′-Diamino Benzidine

DAG- Diacyl Glycerol

DMSO- Dimethyl Sulphoxide

DNA- deoxyribonucleic acid

DOCK3- Dedicator of cyto-kinesis 3

ECM- Extracellular Matrix

EGF- Epidermal Growth Factor

EGFR- Epidermal Growth Factor Receptor

EMT- Epithelial-Mesenchymal Transition

eNOS- Endothelial Nitric Oxide Synthase

FBXW7- F-box/WD-repeat containing protein 7

FCS- Foetal Calf Serum

FGF- Fibroblast growth Factor

GAPDH- Glyceraldehyde 3-phosphate dehydrogenase

GPCR- G-Protein Coupled Receptor

HIV- Human Immunodeficiency Virus

HNSCC- Head and Neck Squamous Cell Carcinoma

HPV- Human Papilloma Virus

IARC- International Agency for Research on Cancer

ILK- Integrin Linked Kinase

JAK- Janus Kinase

LKB1- Liver Kinase B1

MAPK- Mitogen Activated Protein Kinase

MDM2- Mouse Double Minute 2 homolog

MET- Mesenchymal to Epithelial Transition

MMP- Matrix Metalloproteinase

mTOR- Mammalian Target of Rapamycin

NEDD9- Neural precursor cell expressed, developmentally down-regulated 9

NFkB- Nuclear Factor kappa-light-chain-enhancer of activated B cells

NNK- Nicotine-derived Nitrosamine Ketone

NSCLC- Non-small cell lung cancer

OSCC- Oral Squamous Cell Carcinoma

PAK1- p21 protein-activated kinase 1

pAkt S473- Phosphorylated Akt at Serine 473

pAkt T308- Phosphorylated Akt at Threonine 308

PBS- Phosphate Buffered Saline

PCR- Polymerase Chain Reaction

PDK1- Phosphoinositide-dependent Kinase 1

PIP2- Phosphoinositide biphosphate

PIP3- Phosphoinositide 3,4,5 triphosphate

PI3K- Phosphoinositide 3-kinase

PKB- Protein kinase B

PKC- Protein Kinase C

PlGF- Placental Growth Factor

PTEN- Phosphatase and Tensin Homolog

RIPA- Radioimmunoprecipitation Assay

RNA- Ribonucleic Acid

RTK- Receptor Tyrosine Kinase

SCC- Squamous Cell Carcinoma

STAT- Signal Transducer and Activator of Transcription

TBST- Tris-buffered Saline and Tween 20

TGF- Transforming Growth Factor

TME- Tumour Micro-environment

TSC- Tuberous Sclerosis Complex

VEGF-Vascular Endothelial Growth Factor

VEGFR- Vascular Growth Factor Receptor

VPF- Vascular Permeability Factor

WCRF- World Cancer Research Fund

ABSTRACT

The PI3K-Akt signalling pathway is a well-established driver of cancer progression. One key process promoted by Akt phosphorylation is tumour cell motility; however the mechanism of VEGF-induced Akt phosphorylation leading to motility remains poorly understood. Previously, it has been shown that Akt phosphorylation, induced by different factors, causes both stimulation and inhibition of motility in different cell types. However, differential phosphorylation of Akt at T308 and S473 residues by VEGF and its role in head and neck cancer cell motility and progression is unknown. The cell lines investigated in this study exhibited a change in phosphorylation of Akt in response to VEGF. However, in terms of motility, VEGF stimulated oral cancer and its associated cell lines, but not normal keratinocytes or oral mucosal fibroblasts. The addition of a PI3 kinase and mTOR inhibitor, inhibited the phosphorylation of Akt and also effectively blocked VEGF-induced oral cancer cell motility, whereas only the PI3 kinase inhibitor blocked oral cancer associated fibroblast cell motility. This study therefore discloses that two different mechanisms of Akt phosphorylation control the motility potential of different cell lines. Akt phosphorylated at both residues controls oral cancer cell motility.

Tobacco, alcohol and HPV infection are associated with increased risk of HNSCC. However, little is known about the underlying signalling events influencing risk. It was also aimed to investigate the relationship between these risk factors and Akt phosphorylation, to determine prognostic value. VEGF-positive HNSCC biopsies,

with known HPV status, were analysed by immunohistochemistry (IHC) for Akt, phosphorylated at residues S473 and T308. Comparisons between the tissues were carried out using a Mann-Whitney U test. Associations between the variables and continuous immunohistochemical parameters were evaluated with general linear models. Patient characteristics and pAkt IHC score were analysed for possible association with overall survival by Cox proportional hazard models. Immunohistochemistry revealed that cancer patients had significantly higher levels of pAkt T308 than S473 ($P < 0.001$). Smoking and alcohol were found to be independent risk factors for Akt phosphorylation at T308 ($P = 0.022$ and 0.027, respectively). Patients with tumours positive for HPV or pAkt S473 had a poorer prognosis ($P = 0.005$, and 0.004, respectively). Patients who were heavy drinkers were more likely to die than non-drinkers ($P = 0.003$). Patients with low pAkt T308 were more likely to be HPV positive ($P = 0.028$). Non-drinkers were also found to have lower levels of pAkt T308 and were more likely to have tumours positive for HPV than heavy drinkers ($P = 0.044$ and 0.007, respectively). This study suggests different mechanisms of carcinogenesis are initiated by smoking, alcohol and HPV. The resultant data propose higher phosphorylation of Akt at T308 as a reliable biomarker for smoking and alcohol induced HNSCC progression and higher phosphorylation of Akt at S473 as a prognostic factor for HNSCC.

PUBLICATIONS

Islam MR, Jones SJ, Macluskey M, Ellis IR., (2014) Is there a pAkt between VEGF and oral cancer cell migration? Cellular Signalling, 26(6), 1294-1306.

Islam MR, Ellis IR, Cochrane L, Macluskey M, Jones SJ., (2014) Activation of Akt at T308 and S473 in alcohol, tobacco and HPV-induced HNSCC: Is there evidence to support a prognostic or diagnostic role? Experimental Hematology and Oncology, 3(1):25. doi: 10.1186/2162-3619-3-25.

Islam MR, Alkhadar H, Paterson Z, Macluskey M, Jones S, Mossey P, Ellis I., (2011) VEGF and Oral cancer: *Ex-vivo* and *In-vitro* studies, Eur J Cancer; 47. doi:10.1016/S0959-8049(11)72235-3. (Abstract)

Islam MR, Alkhadar H, Paterson Z, Macluskey M, Jones S, Mossey P, Ellis I., (2011) Is there a pAkt between VEGF and motility in oral cancer? f1000, 2, 1511. (Abstract)

Chapter 1 Introduction and Literature Review

1.1 Introduction

State-of-the-art laboratory research and clinical practice has significantly improved therapeutic regimens and early detection of cancer including HNSCC which have enhanced the prospect of cure and progression-free survival. However, patients with late-stage or metastasised cancer often get rewarded just a few months of survival advantage when treated by the latest molecular targeted therapy (Wan *et al.*, 2013). The ability of cancer cells to metastasise from primary tumours contributes to a progressing tumour burden that is circulated across several sites in the body, causing death for many patients. Identifying the cellular and molecular steps that are used by cancer cells during metastasis and their interaction with the microenvironment can establish the basis of new ideas for prognostic, diagnostic and therapeutic approaches that might help restrain cancer metastasis (Wang *et al.*, 2005a, Yamaguchi *et al.*, 2005, Quail and Joyce, 2013). Cell migration and invasion into neighbouring extracellular matrix, the lymphatic system and blood vessels is the critical step in metastasis. Controlling cell migration is critical in cancer treatment (Stracke *et al.*, 1991) . However, the cascade of events leading to cell migration during cancer progression is still poorly understood. The role of the vascular endothelial growth factor (VEGF)-induced PI3K/Akt pathway in oral cancer cell migration, their control and identifying the prognostic value of phosphorylated Akt in HNSCC has been investigated and discussed in this project.

1.2 Cancer

The generic term `cancer' used to denote a group of about 200 diseases sharing common characteristics in which abnormal cells divide uncontrollably and can invade other tissues (Yarbro *et al.*, 2005, Gabriel, 2008). There are over 100 different kinds of cancer. The majority of cancers are named for the organ or type of cell in which they originate- for example, cancer that starts in the head and neck region is called head and neck cancer, cancer that begins in the squamous cells of the skin is called squamous cell carcinoma.

Different types of cancer can be grouped into broader categories. The main categories of cancer comprise (NCI, 2012):

- Carcinoma- cancer that starts in the skin or in tissues that line or cover internal organs.
- Sarcoma- cancer that starts in blood vessels, bone, muscle cartilage, fat or other connective or supportive tissue.
- Leukaemia- cancer that begins in blood-forming tissue such as the bone marrow and initiates production of large numbers of abnormal blood which are released into the blood.
- Lymphoma and myeloma- cancers that start in the immune system.
- Central nervous system cancer- that starts in the cells of the brain and spinal cord.

All cancers start in cells, the body's fundamental unit of life. To understand cancer, it is helpful to be familiar with what happens when normal cells become cancer cells.

The body comprises numerous types of cells. These cells grow and divide in a controlled manner to create more cells as they are essential to keep the body healthy. When cells become older or injured, they die and are restored with new cells.

However, occasionally this orderly system becomes erroneous. DNA of a cell can become changed, generating mutations that influence normal cell growth and division. When this occurs, cells do not die when they should and new cells are made when the body does not require them. These additional cells may form a mass of tissue called a tumour (Ruddon, 2007, NCI, 2012).

Tumours can be Benign and Malignant.

- Benign tumours- Cells in benign tumours do not spread to other parts of the body. They can often be removed and in most cases, they do not reappear.
- Malignant tumours- these cancerous tumours can invade local tissues and spread to other parts of the body.

The spread of the cancerous cells expands outward from the original tumour and has been expressed as similar to the appearance of a crab. This is the basis of the term ‘cancer’, which was originated from the Latin meaning ‘crab’ (Walter, 1977).

The earlier a cancer is identified, the less likely it is to spread, and so the more positive the prognosis for the individual (DoH, 2000).

1.2.1 Head and Neck cancer

The majority of head and neck cancer begin in the cells lining the open cavities of the head and neck, such as the mouth, throat, sinuses and the nasal cavity. Other type of head and neck cancer can appear in the salivary glands. Cancers of the brain and thyroid are not categorised as head and neck cancer (Shah *et al.*, 2001, Brockstein and Gregory, 2002). Head and neck squamous cell carcinomas (HNSCC) comprise the vast majority of head and neck cancers and appear from mucosal surfaces throughout this anatomic region. They comprise tumours of the oral cavity, nasopharynx, oropharynx, hypopharynx, larynx, nasal cavities and paranasal sinuses (Figure 1.1) (Barnes *et al.*, 2005).

These cancers frequently spread to the lymph nodes of the neck, and this is often the first indication of the disease at the time of diagnosis (Samson *et al.*, 2010).

1.2.1.1 Oral cavity

The oral cavity consists of the lips, the front two-thirds of the tongue, the gums, the lining within the cheeks and lips, the floor of the mouth under the tongue, the hard palate and the gum at the rear of the wisdom teeth (Hiatt and Gartner, 2009).

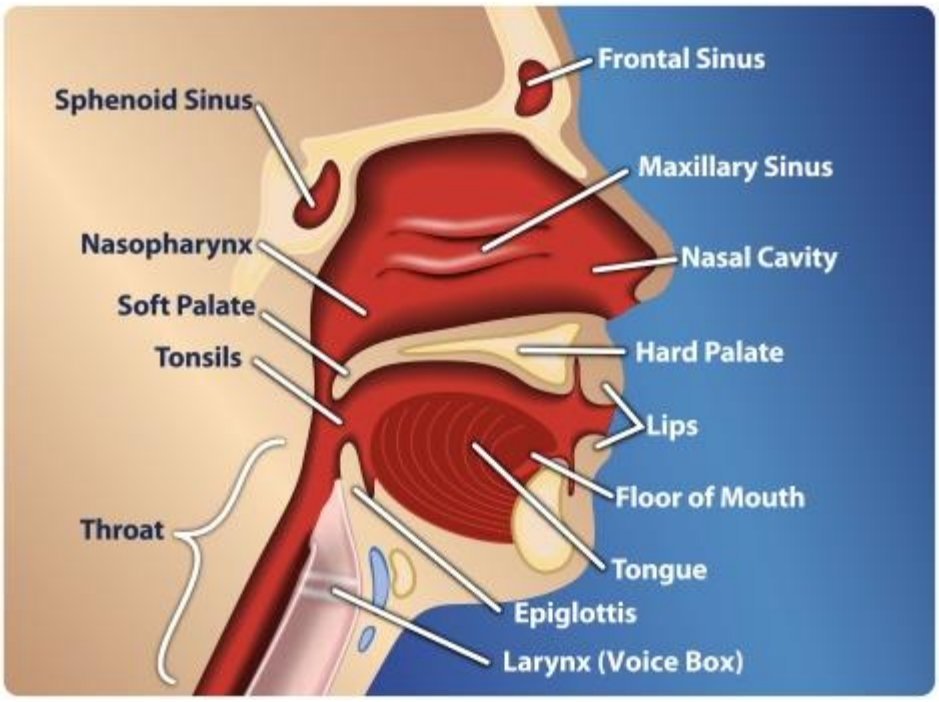


Figure 1.1 The region of head and neck.

The figure shows different parts of the head and neck including nasal cavity, lips, tongue, floor of the mouth, nasopharynx and larynx (Eisenberg, 2010).

1.2.1.2 Pharyngeal

The pharynx is a hollow tube about 5 inches long that leads from the back of the nose to the oesophagus. It has three parts:

Nasopharynx: The upper fraction of the pharynx, behind the nose.

Oropharynx: The central part of the pharynx, extends from the soft palate to the superior border of the epiglottis. Oropharynx is surrounded superiorly by the soft palate, laterally by the palatoglossal arches and inferiorly by the tongue.

Hypopharynx: The inferior part of the pharynx (Norton, 2007, Hiatt and Gartner, 2009)

1.2.1.3 Laryngeal

The larynx is also called the voicebox, a short passageway shaped by cartilage, just underneath the pharynx in the neck. The larynx contains the vocal cords. It also contains a small piece of tissue, called the epiglottis, which shifts to cover the larynx to stop food from entering the air passages (Norton, 2007).

1.2.1.4 Paranasal sinuses and nasal cavity

The paranasal sinuses are small void spaces in the bones of the head encircling the nose. The nasal cavity is the hollow space within the nose.

1.2.1.5 Salivary glands

The major salivary glands are in the floor of the mouth and next to the jawbone. The salivary glands generate saliva (Norton, 2007, Hiatt and Gartner, 2009).

1.2.2 Oral cavity

The oral cavity (Figure 1.2) is oval shaped and is divided into the oral vestibule and the oral cavity proper (Snow *et al.*, 2009). It is connected by the lips anteriorly, the palate superiorly, the cheeks laterally, the oropharynx posteriorly and the floor of the mouth inferiorly (Probst *et al.*, 2006). The bony base of the oral cavity is characterised by the maxillary and mandibular bones.

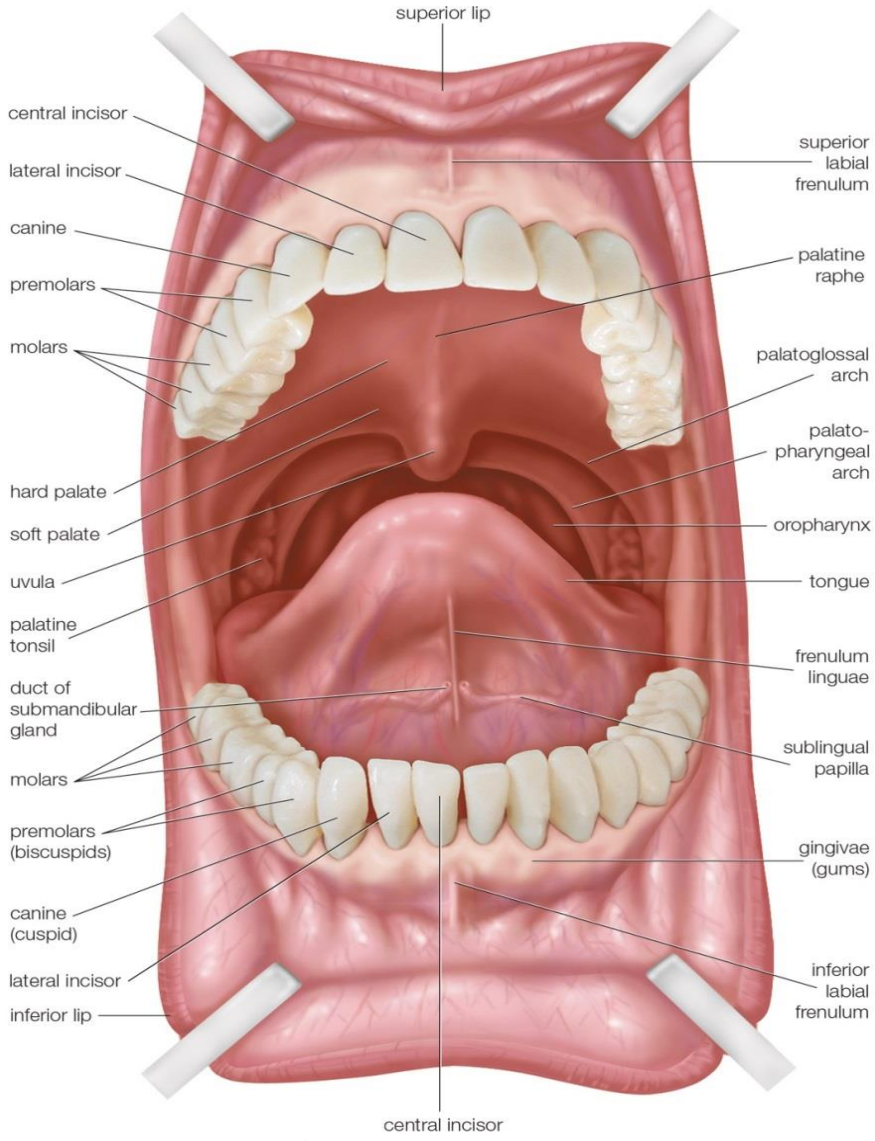


Figure 1.2 Graphical representation of oral cavity.

It is showing hard palate, soft palate, tongue, uvula, tonsil, submandibular gland, oropharynx, lips and teeth (Dworken et al., 2012).

The oral cavity consists of the lips, teeth, hard palate, cheek mucosa, tongue, floor of the mouth, gingivae and retromolar trigone. The major salivary glands are not part of oral cavity. The palatine tonsils, tongue base, soft palate and posterior pharyngeal

walls are components of the oropharynx which are not part of the oral cavity (Berkovitz *et al.*, 2002, Shah *et al.*, 2003, Edge, 2010).

1.2.2.1 Normal histology of oral cavity

The oral cavity is lined by the oral mucosa, a specialised tissue. This tissue is designed to offer defence to the body from infection and it is able to produce secretions such as mucus (Squier and Brogden, 2011). The oral mucosa (Figure 1.3) is comprised of stratified squamous epithelium, basement membrane, the lamina propria and submucosa. No hair follicles or sweat glands present in oral mucosa. It contains many sensory receptors including the taste receptors of the tongue. Keratinisation occurs in areas most exposed to mastication like hard palate, gingiva and dorsum of tongue (Shah *et al.*, 2003).

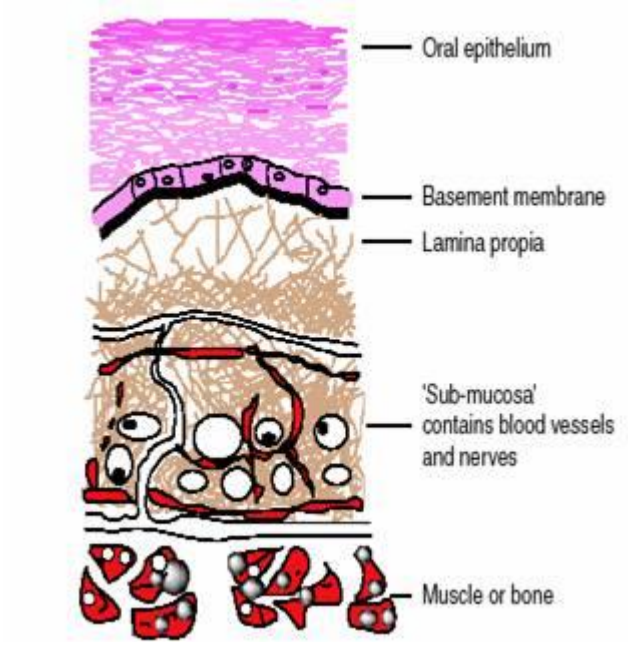


Figure 1.3 Cross section of oral mucosa.
It is comprised of epithelium, basement membrane, lamina propria and sub mucosa (Shojaei, 1998).

The lamina propria is composed of loose connective tissue, mucous glands and serous minor salivary type glands. The submucosa has collagenous, dense, fibrous tissue (Pernick, 2012).

1.2.3 Oral cancer

Oral cancer, a subtype of head and neck cancer, is any cancerous tissue growth located in the oral cavity (Werning 2007). It may appear as a primary lesion originating in any of the oral tissues, by metastasis from a remote site of origin, or by expansion of an adjacent anatomic structure, such as the nasal cavity or the maxillary sinus (Pernick, 2012). An oral tumour may develop in any of the tissues of the mouth and may be of various histological types e.g., teratoma, adenocarcinoma originating from a major or minor salivary gland, lymphoma from tonsillar or other lymphoid tissue, or melanoma from the pigment generating cells of the oral mucosa. There are a number of different types of oral cancers, but about 90% are squamous cell carcinomas (OCF, 2012) deriving in the tissues that line the mouth and lips. These are malignant and likely to spread quickly. Oral or mouth cancer most commonly involves the tongue. It may also occur on the floor of the mouth, cheek lining, gingiva (gums), lips, or palate (roof of the mouth)(Werning, 2007).

1.2.4 Clinical presentations of oral cancer

Ulceration and indurated margin are two very characteristic features of almost all oral cancer, except those in the earliest stages. However there are certain variations in different sites (Bagan *et al.*, 2010).

Lip cancer usually refers to the cancer of vermilion border with the lower lip (Figure 1.4) being much more frequently affected than the upper lip. Vermilion border cancers in contrast to oral mucosa cancer often have their origin in a tissue which is altered by ageing or UV radiation: actinic keratosis and elastosis. This assumes a paler colour, which characterised by narrowing of vermilion border. Cancer of the skin has more similarity to this cancer of modified mucosa and pre cancer in the form of actinic keratosis than that of oral mucosa (Pindborg *et al.*, 1997, Barnes *et al.*, 2005).

Figure 1.4 Lip cancer.

A ulcerated area with indurated and firm margin (Reifman, 2012).

Cancer of the labial commissures are often associated with candidal infection and preceded by nodular leukoplakia.

The majority of buccal mucosa cancers are located posteriorly. Sometimes the cancer extends into the upper or lower sulcus. The buccal mucosa is the major location of oral cancer in some areas of South and South-eastern Asia. This may be due to high occurrence of betel quid chewing with tobacco.

Cancer of the floor of the mouth is often sited anteriorly near or in the midline (Figure 1.5). In advanced cases, adjacent structures such as extension into tongue are common. Leukoplakia(pre-cancerous white patches) or erythroplakia (pre-cancerous red patches) may also be present (Pindborg *et al.*, 1997, Shah *et al.*, 2003, Barnes *et al.*, 2005).

Figure 1.5 Cancer of the floor of the mouth.

A sharply defined margin and raw ulcerated area (Levison *et al.*, 2008).

Tongue cancer often is exophytic and linked with ulceration (Figure1. 6). Sometimes the only presentation of carcinoma is leukoplakia in tongue cancer. The main location of tumour growth is in the lateral border and ventral surface of anterior tongue (Pindborg *et al.*, 1997, Neville *et al.*, 2002).

Figure 1.6 Cancer of lateral border of tongue.

Red and white and warty growth enclosed by leukoplakia (Pindborg *et al.*, 1997).

Palatal cancer develops as swelling that later ulcerates with deep growth (Figure 1.7). Reverse smokers (who smokes a cigarette from the lit end) are at high risk of hard palate cancer which develops as an ulcer lateral to the midline (Bagan *et al.*, 2010).

Figure 1.7 Mouth cancer on hard palate.

Squamous cell carcinoma on the hard palate characterised by growing mass (Sadeghi, 2015).

The term gum cancer is commonly used to describe both cancer in gingiva and edentulous alveolar ridge, although the mucosal structure of both the sites is different. The carcinoma begins as ulceration, often associated with leukoplakia at either site (Figure 1.8) (Pindborg *et al.*, 1997, Barnes *et al.*, 2005).

Figure 1.8 Cancer of the gingiva.

Ulceration with leukoplakia (Buquot, 1992).

Cancer of the vermilion border of lower lip has a lower tendency to produce cervical lymph node metastases. Even though the cancer of the tongue and floor of the mouth are smaller in size, they have a high frequency of spreading to cervical nodes. Cancer located close to or extending to the midline may spread to contra lateral or bilateral lymph nodes but usually to the ipsilateral side of the neck (Pindborg *et al.*, 1997).

1.2.5 Oral pre-cancerous lesion

Histologically oral pre-cancerous lesions are divided into three classes:

i. Epithelial hyperplasia

ii. Squamous epithelial dysplasia

iii. Squamous cell carcinoma *in situ*

1.2.5. i Epithelial hyperplasia

Cytologically no changes occur in the hyperplasia but architecturally they are characterised by thickened epithelium, hyperkeratosis and normal maturation (Figure 1.9) (Speight, 2007).

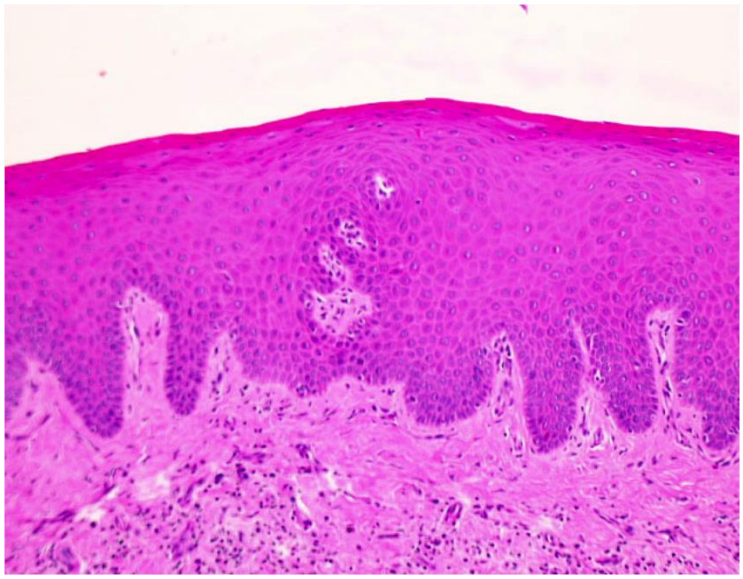


Figure 1.9 Epithelial hyperplasia.

Thickened epithelial layer with normal maturation (Speight, 2007).

1.2.5. ii Squamous epithelial dysplasia

Squamous epithelial dysplasia is a pre-cancerous condition of stratified squamous epithelium. It is characterised by cellular atypia and loss of normal cell maturation and stratification short of carcinoma *in situ*. The general disorder of the epithelium is designated as dysplasia and the possibility for progressing to invasive carcinoma increases with the severity of dysplasia (Pindborg *et al.*, 1997, Speight, 2007). Table 1 lists the changes that may occur in epithelial dysplasia.

Table 1 Histological transformations to the diagnosis of epithelial dysplasia

Architecture	**Cytology**
Drop-shaped rete-ridges Polarity loss of basal cells Irregularity of epithelial stratification Number of mitotic figures increases Mitotic figures present in the superficial half of the epithelium Dyskeratosis (Premature keratinisation in single cells) Keratin pearls with rete pegs Loss of intercellular adherence	Abnormal form (Atypical) of mitotic figures Anisonucleosis(abnormal variation of nuclear size) Nuclear pleaomorphism (abnormal variation in nuclear shape) Anisocytosis (abnormal variation in cellular size) Cellular pleomorphism (abnormal variation in cellular shape) Nuclear-cytoplasmic ratio increases Increased nuclear size Nuclear hyperchromatism Increased number and size of nucleoli

Note. The more aggressive or more numerous the changes are, the more severe the grade of dysplasia (Speight, 2007, Ramadas *et al.*, 2012).

Any degree of dysplasia (Figure 1.10-1.12), even a mild form, designates an increased risk for the patient. Severe dysplasia signifies that there is a very high risk of the consequent growth of cancer. Dysplasia is often seen synchronised with invasive carcinoma.

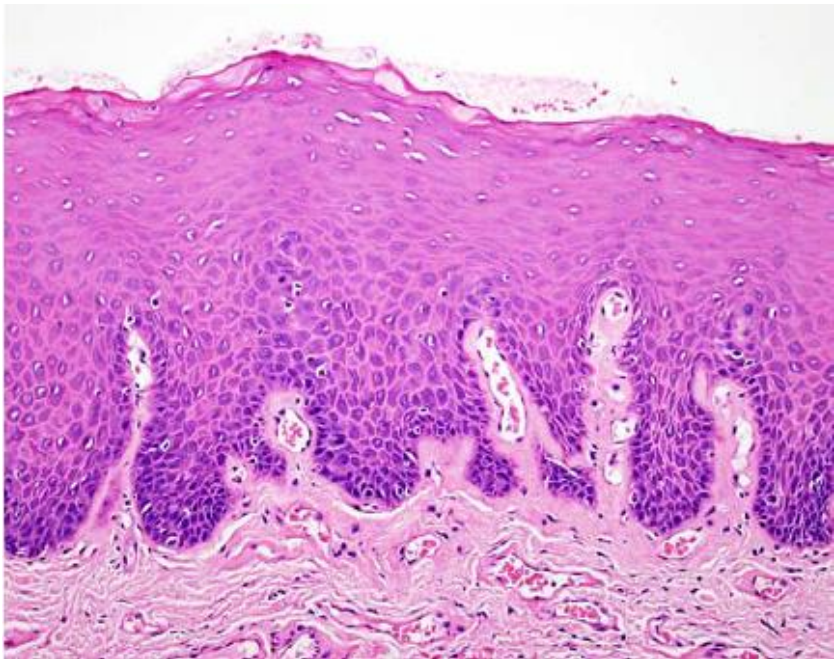


Figure 1.10 Mild dysplasia.

Basal cell atypia including hyperchromatism and pleomorphism is common without any architectural changes. Stratification is also normal (Speight, 2007).

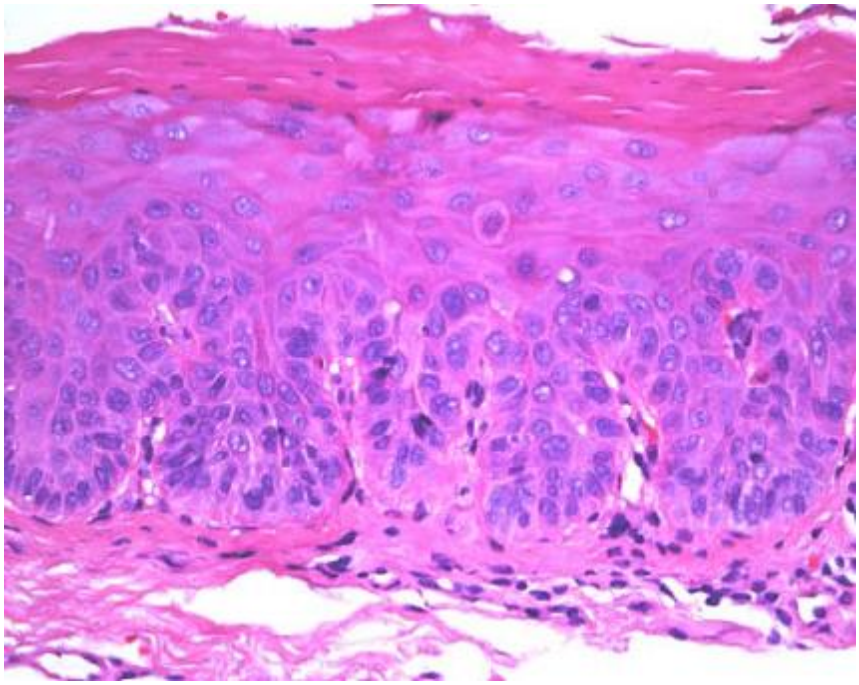


Figure 1.11 Moderate dysplasia.

A considerable cellular atypia extends into the middle third of the epithelium (Speight, 2007).

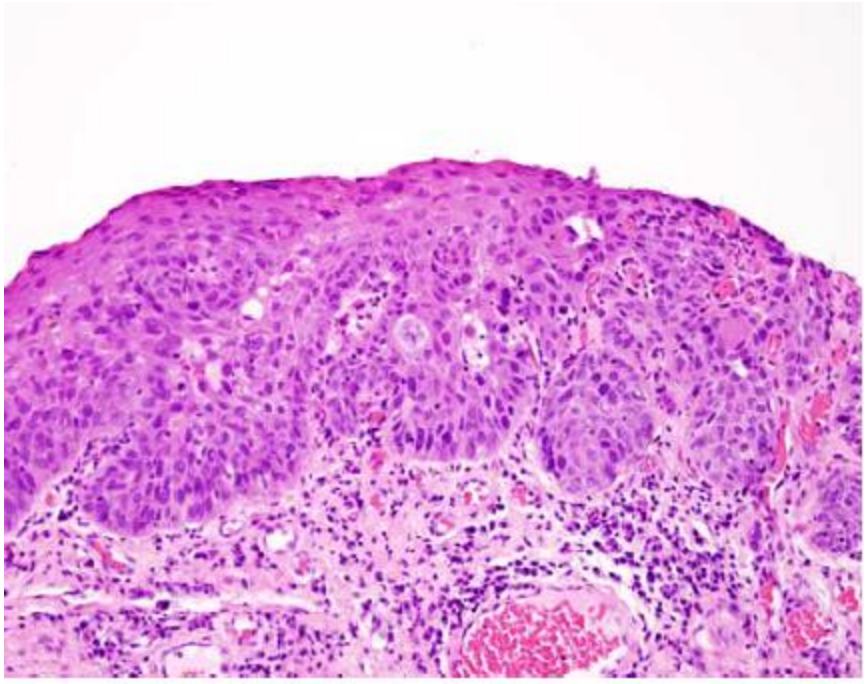


Figure 1.12 Severe dysplasia.

Cellular atypia spreads into the upper third of the epithelium. Basal cell hyperplasia is common. Disruption of the normal architecture of the epithelium and spherical rete pegs are noticeable (Speight, 2007).

1.2.5. iii Squamous cell carcinoma in situ

Carcinoma *in situ* is a lesion in which full thickness or almost the full thickness of squamous epithelium demonstrates the cellular characteristics of carcinoma without stromal invasion (Figure 1.13).

Severe grades of dysplasia are amalgamated into lesions routinely regarded as carcinoma *in situ* in which the whole or almost the whole of the epithelium is involved.

However, differentiating between severe dysplasia and carcinoma *in situ* is often tricky and does not appear to be of practical value in the case of the oral mucosa (Pindborg *et al.*, 1997).

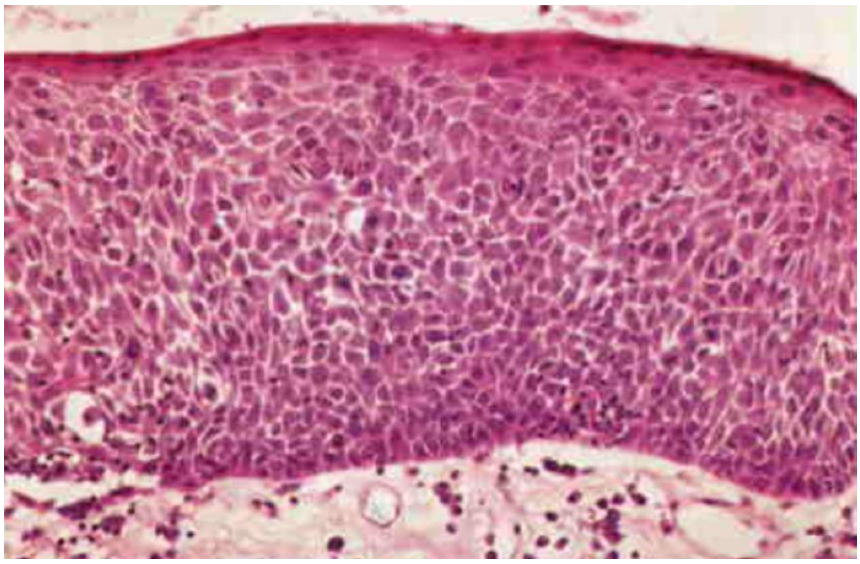


Figure 1.13 Carcinoma *in situ*.

Hyperproliferation of epithelial cells. The cells have not acquired the required genetic lesions that would assist basement membrane diffusion and invasion (Regezi and Jordan, 2001).

1.2.6 Oral Carcinomas

According to WHO tumour classification (2005) Oral carcinomas can be classified into the following types (Barnes *et al.,* 2005):

i. Squamous Cell Carcinoma
ii. Verrucous Carcinoma
iii. Basaloid Squamous Cell Carcinoma
iv. Acantholytic Squamous Cell Carcinoma
v. Spindle Cell Carcinoma
vi. Adenosquamous Carcinoma
vii. Papillary Squamous Cell Carcinoma
viii. Carcinoma Cuniculatum

1.2.6.i Squamous cell carcinoma

Squamous cell carcinoma is the malignant neoplasm of epithelium displaying squamous differentiation as distinguished by the development of keratin and/or the existence of intercellular bridges (Pindborg *et al.,* 1997).

To establish a prognosis for the patient or an indicator for the most successful treatment and in an attempt to forecast their aggressiveness it has been usual to grade this neoplasm. This grading is based on the subjective measurement of the degree of keratinisation, cellular and nuclear pleomorphism and mitotic activity (Pindborg *et al.,* 1997, Bhargava *et al.,* 2010). The grades are:

- Grade 1: Well differentiated carcinoma that closely resemble to normal squamous epithelial lining of the oral mucosa, histologically and cytologically. Keratinisation is the outstanding feature in this grade where there is varying extent of basal and squamous cells with intercellular bridges. Cellular and nuclear pleomorphism is negligible, atypical mitoses and multinucleated epithelial cells are extremely rare. A small number of mitotic figures are also seen (Pindborg *et al.*, 1997, Woolgar and Triantafyllou, 2009, Bhargava *et al.*, 2010) (Figure 1.14).

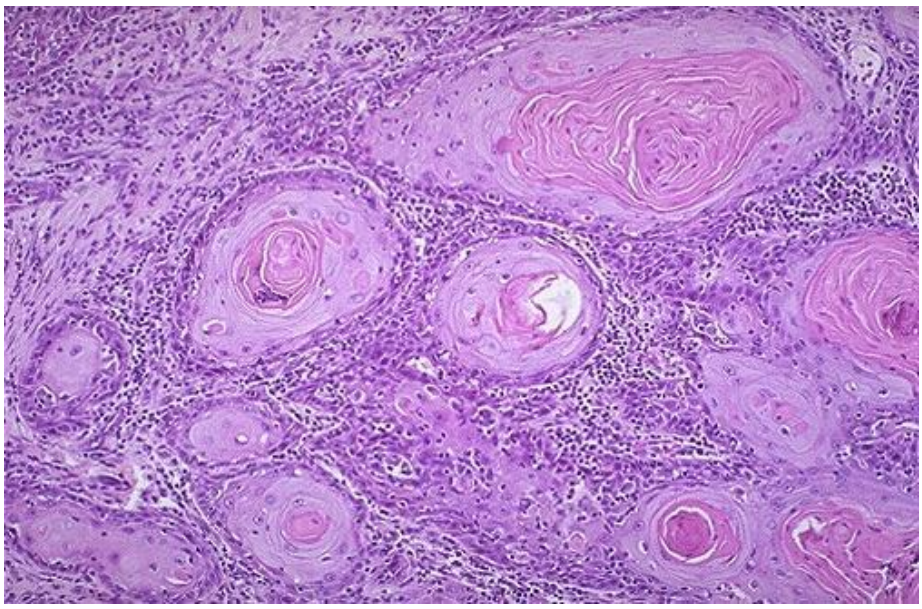


Figure 1.14 Well differentiated squamous cell carcinoma.

Infiltrating nests and sheets of keratinising cells are presents (IARC, 2015) .

- Grade 2: Moderately differentiated neoplasm which has less keratinisation and more cellular and nuclear pleomorphism than well-differentiated squamous cell carcinoma. There are more mitotic figures with some

abnormal form and less obvious intercellular bridges are also present (Pindborg *et al.*, 1997, Woolgar and Triantafyllou, 2009, Bhargava *et al.*, 2010) (Figure 1.15).

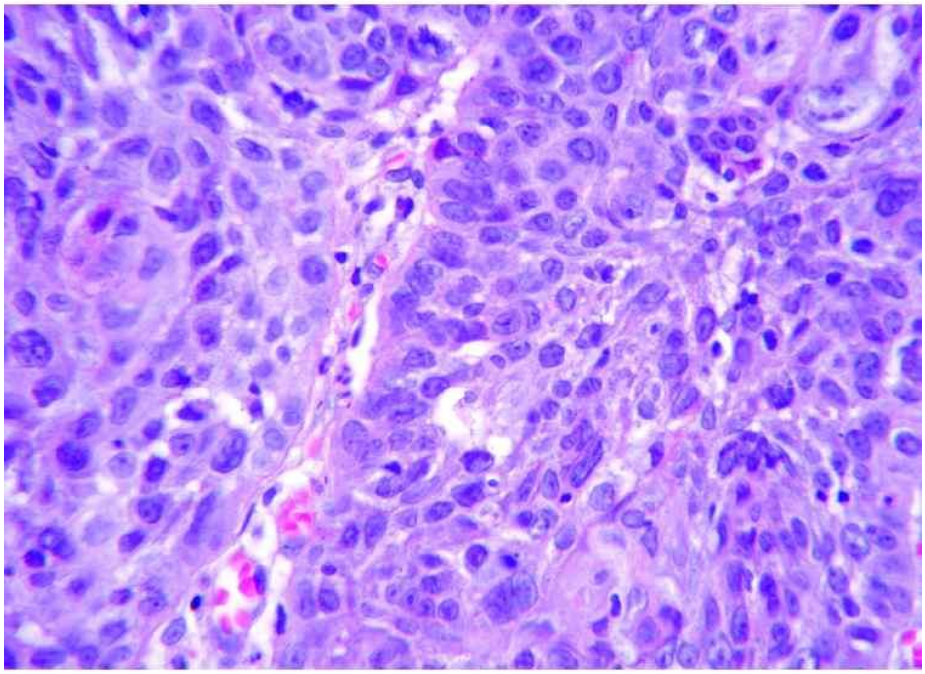


Figure 1.15 Moderately differentiated squamous cell carcinoma.

Characterised by limited keratin configuration (IARC, 2015).

- Grade 3: Poorly differentiated carcinoma which has rare keratinisation and extremely scarce intercellular bridges. Mitotic activity and multinucleated cells are frequent. Cellular and nuclear pleomorphism and atypical mitoses are obvious (Pindborg *et al.*, 1997, Woolgar and Triantafyllou, 2009, Bhargava *et al.*, 2010) (Figure 1.16).

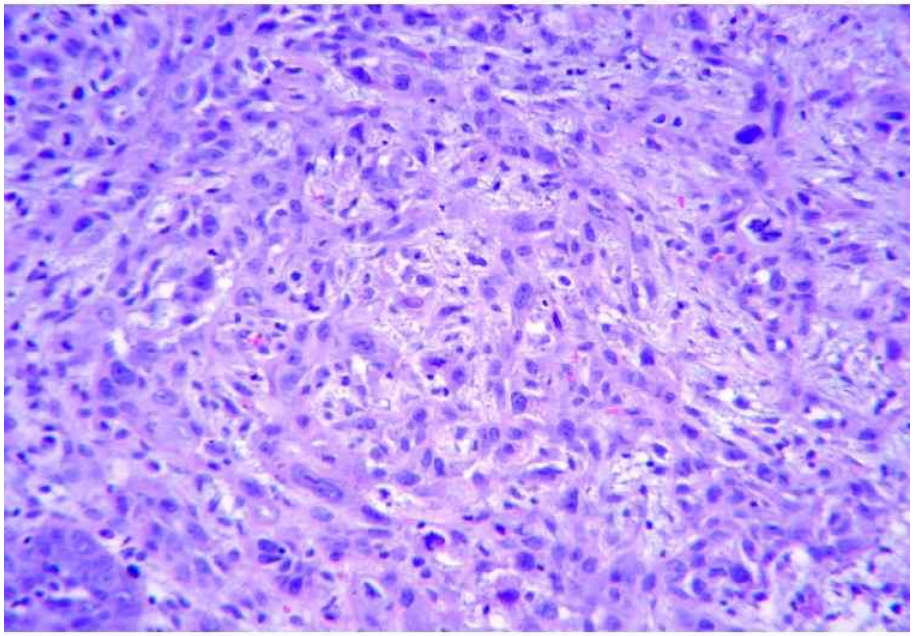


Figure 1.16 Poorly differentiated oral squamous cell carcinoma.
Non-keratinising sheets of infiltrating carcinoma (IARC, 2015).

There are no known differences between oral squamous cell carcinoma and those found elsewhere in terms of capacity to extend by invasion into neighbouring tissues. Tumour infiltration into bone and intra-vascular spread is common as much of the oral mucosa is closely related to blood vessels and underlying bone. Invasion may occur sometimes by dissociated individual cancer cells and in the form of solid sheets, cords or islands of malignant cells. The basement membrane may be more or less discrete or completely absent. The term microinvasive squamous cell carcinoma is often used when invasion by islands or strands of squamous carcinoma engages only the most superficial regions of primary connective tissue, just below the basement membrane (Pindborg *et al.*, 1997, Barnes *et al.*, 2005, Wang *et al.*, 2005b, Harrison *et al.*, 2008).

When squamous cell carcinoma infiltrates through stromal tissue, this may show an uneven chronic inflammatory reaction and in some instances a desmoplastic response is aggravated. Extensive spreading through lymphatics and blood vessel also occurs (Pindborg *et al.*, 1997, Barnes *et al.*, 2005).

1.2.6.ii Verrucous Carcinoma

Verrucous carcinoma(VC) is exophytic, warty, slowly growing variant of well differentiated keratinising epithelium with minimal atypia and limited pushing margins at its interface with underlying connective tissues (Pindborg *et al.*, 1997, Barnes *et al.*, 2005) (Figure 1.17). Hyperplastic well-differentiated stratified epithelium is arranged into bulbous rete-ridges that demonstrate no or little cytological atypia or mitotic activity. A considerable endophytic component may be present and the invading boundary is typically below the level of the surrounding mucosa. The advancing epithelial margin is wide and the basement membrane is usually undamaged. Strong inflammatory cell reaction can be seen in adjacent connective tissues and local destruction of the connective tissues occurs in advance of the deep epithelial margin. Pure verrucous carcinoma's growth is normally slow and metastasis is very rare. However, hybrid VC (non-verruous SCC that arises synchronously with VC) has the potential to metastasise. Although very uncommon, 75% of all cases are found in the oral cavity and usually occurs in older males

(Grinspan and Abulafia, 1979, Tornes *et al.*, 1985, Pindborg *et al.*, 1997, Barnes *et al.*, 2005).

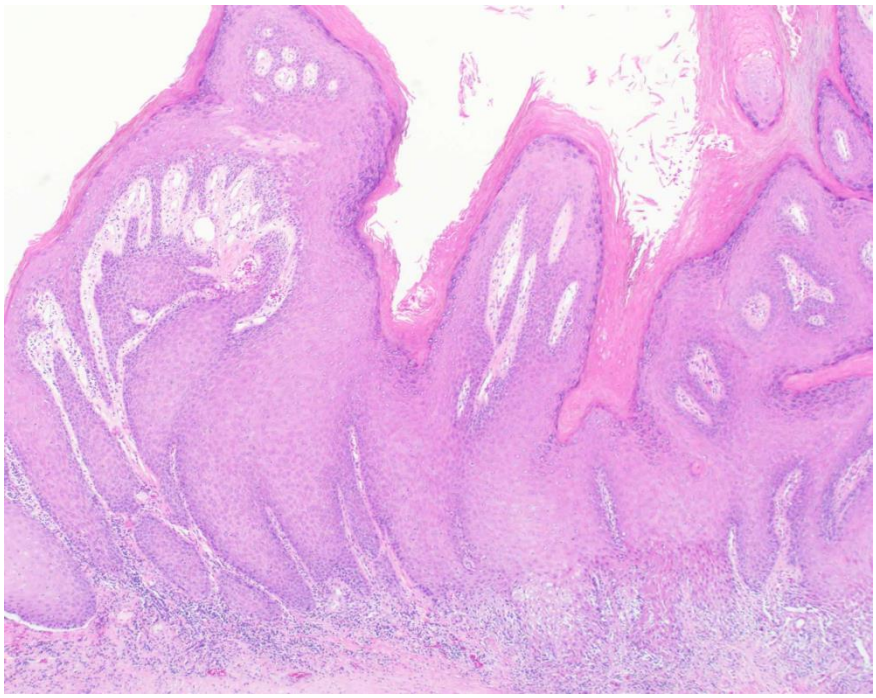


Figure 1.17 Verrucous carcinoma.
Exophytic hyperkeratinised SCC with atypical cytology and extended epithelial rete-ridges (Barnes *et al.*, 2005).

1.2.6.iii Basaloid Squamous Cell Carcinoma

Basaloid squamous cell carcinoma is an aggressive and high grade variant of SCC with a mixed composition of basaloid and squamous cells (Pindborg *et al.*, 1997, Barnes *et al.*, 2005)(Figure 1.18). The basaloid components of this type of oral carcinoma are comprised of hyperchromatic nuclei and scant cytoplasm which are crowded together into lobulated sheets or strands. These sheets are usually

connected focally to the surface epithelium. Palisaded cells are often seen at the periphery of the lobules. More centrally, sometimes there may be cystic spaces containing mucin-like materials and focal squamous differentiation. Mitotic figures are common with variable hyalinisation of surrounding stroma and chronic inflammatory cell infiltration. Larynx, hypopharynx and base of the tongue are the most common areas where basaloid carcinoma occurs (Banks *et al.*, 1992, Barnes *et al.*, 1996, Pindborg *et al.*, 1997, Barnes *et al.*, 2005).

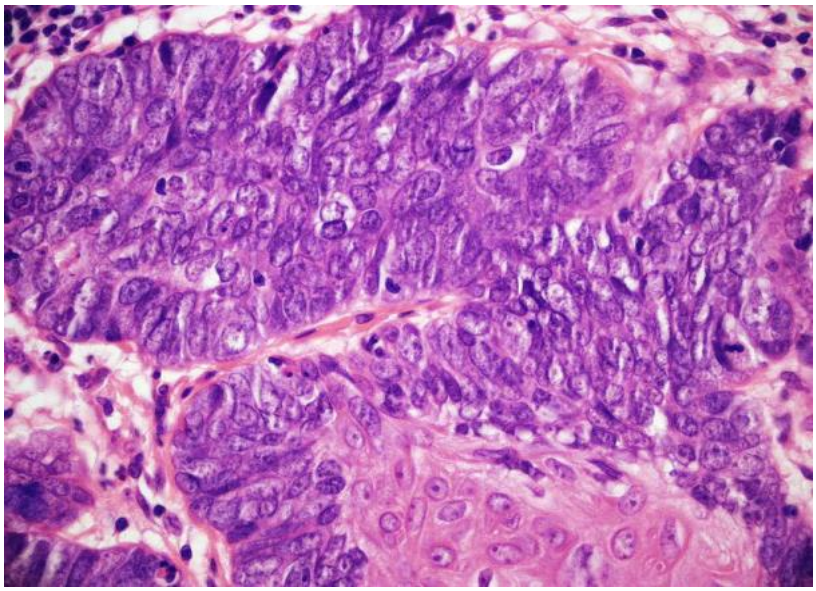


Figure 1.18 Basaloid squamous cell carcinoma.
Small cell with hyperchromatic nuclei demonstrate peripheral palisading and surround areas of cystic necrosis (Pindborg *et al.*, 1997).

1.2.6.iv Acantholytic Squamous Cell Carcinoma

Pseudoglandular spaces or lumina containing squamous cell carcinoma are termed acantholytic squamous cell carcinoma (Figure 1.19), also known as pseudoglandular

squamous carcinoma. Keratinocyte acantholysis and degeneration within islands of a SCC are the major characteristics of this variant. It appears as pseudoadenocarcinoma, but there is no sign of glandular differentiation or of secretory products or functions (Pindborg *et al.*, 1997, Barnes *et al.*, 2005).

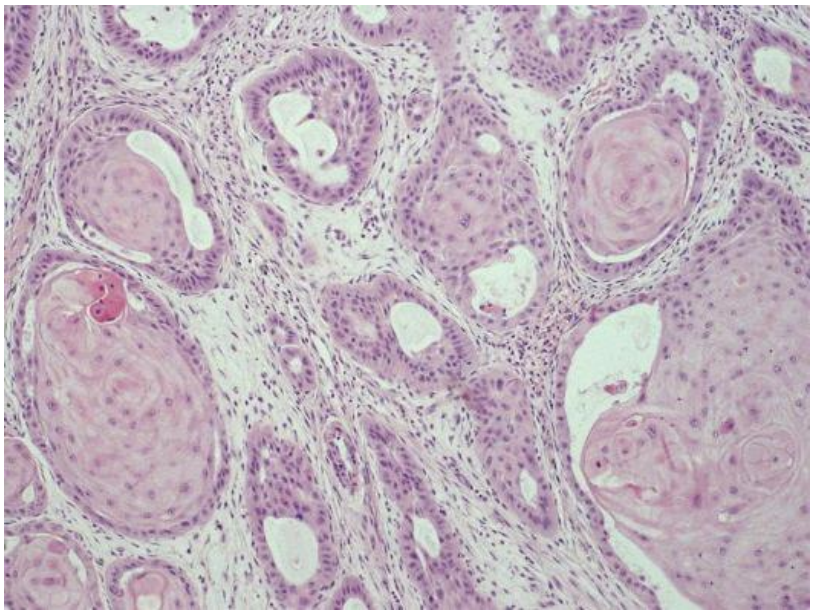


Figure 1.19 Acantholytic squamous cell carcinoma.
Characterised by pseudoglandular spaces and acantholysis (Pindborg *et al.*, 1997).

1.2.6.v Spindle Cell Carcinoma

This type of carcinoma contains some elements that resemble squamous cell carcinoma which are associated with spindle cell components (Figure 1.20). This type of carcinoma is also called sarcomatoid carcinoma. The malignant spindle-shaped cells are epithelial in origin and derived from squamous cell sections of the carcinoma. Most of the carcinoma contains thin elongated cells with occasional pleomorphic cell and frequent mitotic figures. Spindle cell carcinoma should be

distinguished very carefully from squamous cell carcinoma that has activated stromal fibroblastic proliferation (Pindborg *et al.*, 1997, Barnes *et al.*, 2005).

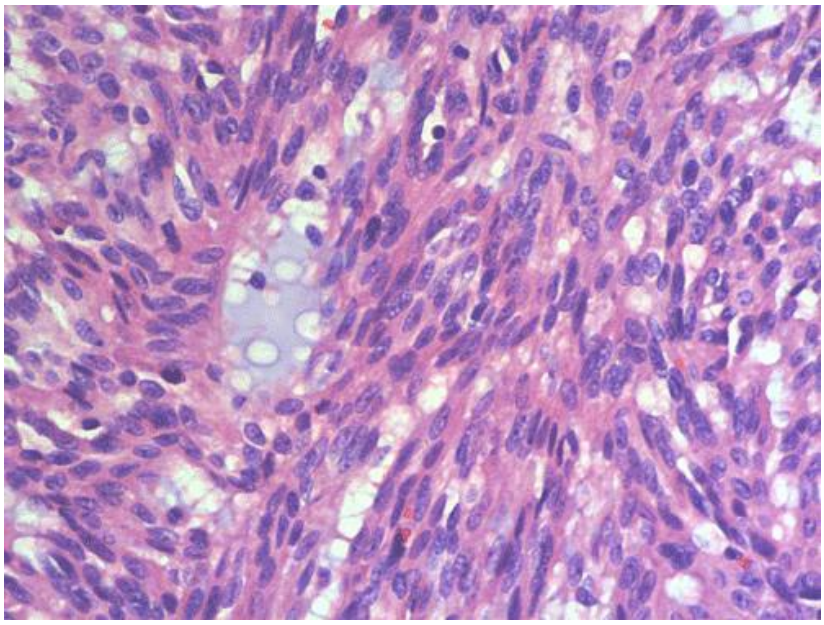


Figure 1.20 Spindle cell carcinoma.
Characterised by thin elongated cells (Barnes *et al.*, 2005).

1.2.6.vi Adenosquamous Carcinoma

Adenosquamous carcinoma is a malignant carcinoma which is histologically characterised by both adenocarcinoma and squamous cell carcinoma (Figure 1.21). The tumour may appear from the surface epithelium of minor salivary glands or from the ducts of the glands. The component found as squamous cell carcinoma may be *in situ* or invasive. On the other hand, adenocarcinomatous components may include glandular structures lined by columnar, basaloid and mucin-secreting cells (Barnes *et al.*, 2005, Fonseca *et al.*, 2012).

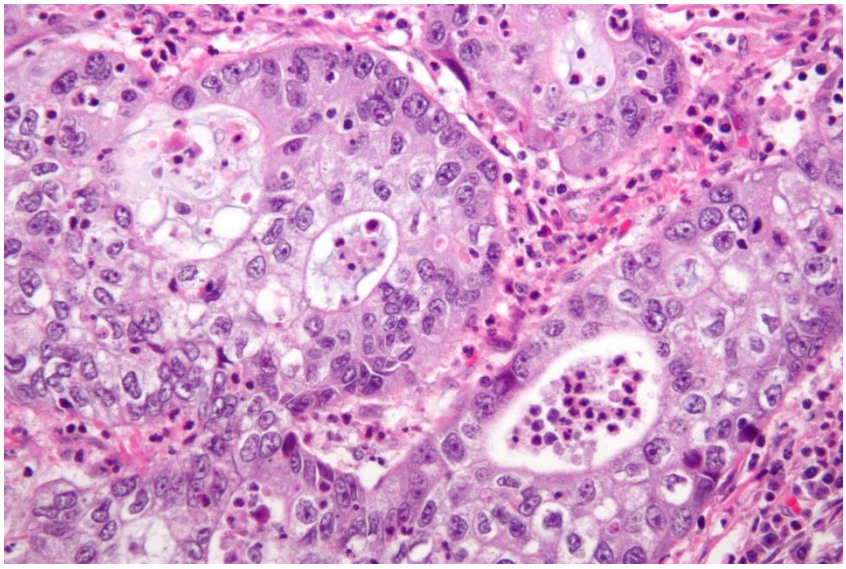


Figure 1.21 Adenosquamous carcinoma.

Characterised by both adenocarcinoma and squamous cell carcinoma (Pindborg *et al.*, 1997).

1.2.6.vii Papillary Squamous Cell Carcinoma

Papillary SCC is a distinctive variant of squamous cell carcinoma characterised by exopphytic papillary(filliform) growth pattern.These filliform growth have thin fibrovascular core covered by neoplastic, immature basaloid cells or more pleomorphic cells (Figure 1.22) . Larynx and hypopharyns are the most common site of this variant of SCC (Barnes *et al.*, 2005).

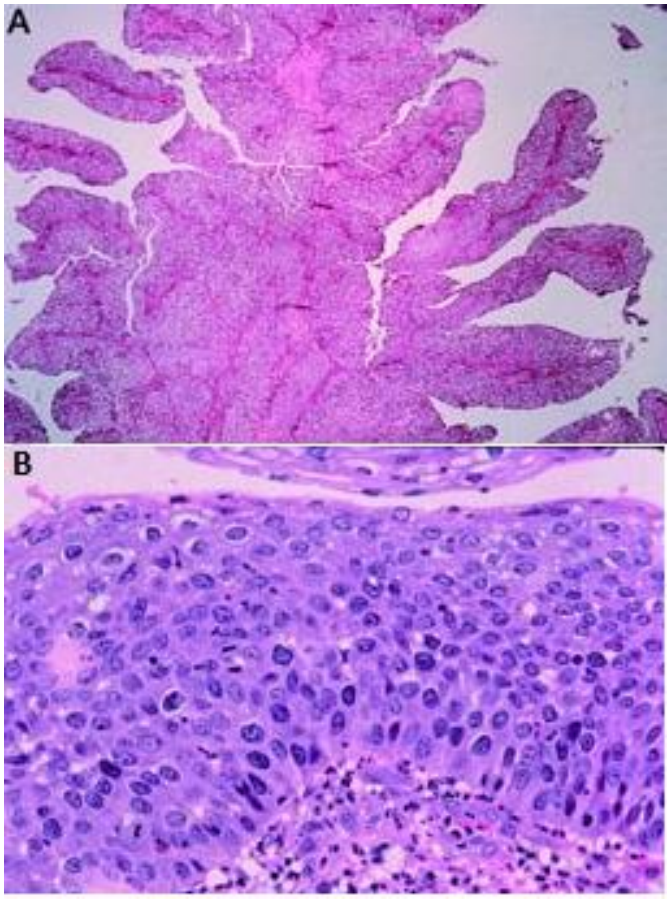


Figure 1.22 Papillary Squamous Cell Carcinoma.

A, Papillary growth with finger-like projections and distinguishable fibrovascular core. B, Markedly pleomorphic cells (Wenig, 2002).

1.2.6.viii Carcinoma Cuniculatum

This is a rare variant of oral cancer which infiltrates deeply into the bone. The keratin filled crypts are surrounded by well-differentiated squamous epithelium along with broad epithelial processes (Figure 1.23). Tumour cells exhibit frequent mitosis and mild cytologic atypia in this type of carcinoma (Allon *et al.*, 2002, Barnes *et al.*, 2005).

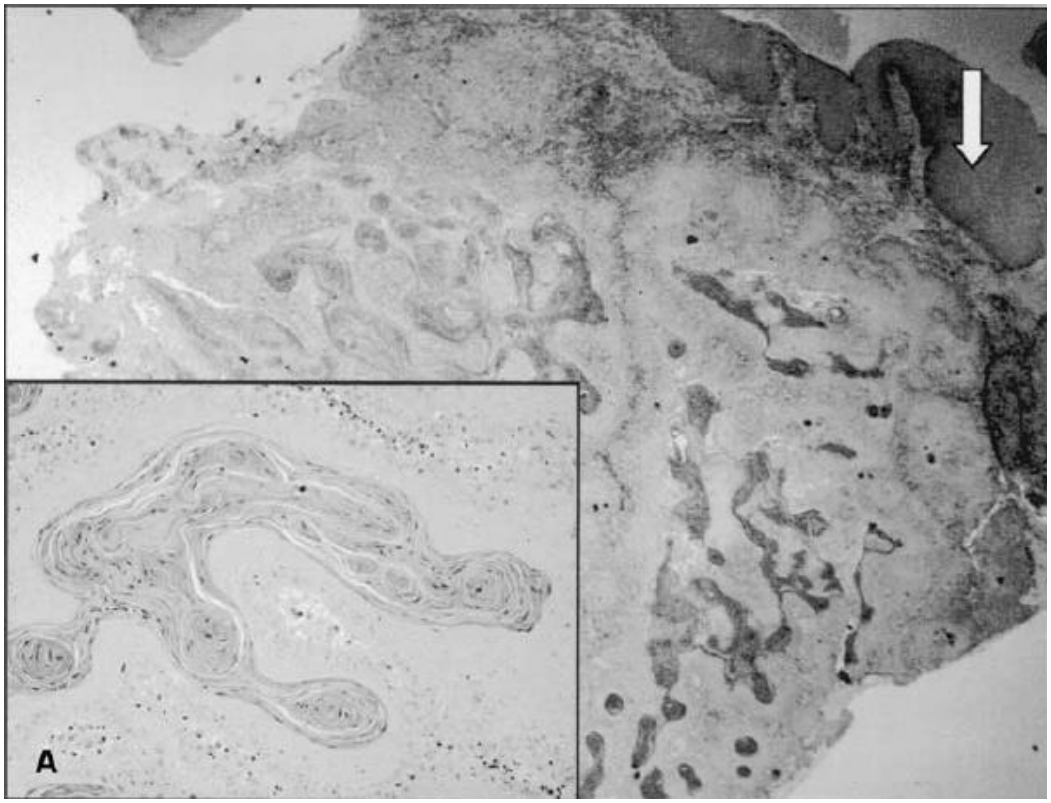


Figure 1.23 Carcinoma Cuniculatum.

Invasive tumour underneath the oral epithelium (arrow) with keratin filled crypts surrounded by well-differentiated squamous cell epithelium (A) (Allon *et al.*, 2002)

1.3 TNM staging system and datasets for reporting oral cancer

Measurement of the mass of the primary tumour (T), the number of local lymph node metastasis (N) and the incidence of distant metastasis(M), collectively called TNM system are broadly used to describe the degree of tumour load and decide treatment options for patients with oral squamous cell carcinoma (Deschler and Day, 2008). Table 2 (Deschler and Day, 2008) describes the TNM staging system for head and neck cancer.

Table 2 TNM staging system of head and neck cancer

Tumour staging by site (Oral cavity)	
Tx	Primary tumour cannot be assessed
T0	There is no evidence of primary tumour
Tis	Carcinoma is in situ.
T1	Tumour is 2 cm or less in greatest dimension
T2	Tumour is more than 2 cm but not greater than 4 cm in greatest dimension
T3	Tumour is more than 4 cm in greatest dimension
T4(lip)	Tumour invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin of face—i.e., chin or nose
T4a	Oral tumour invades adjacent structures (e.g., through cavity) cortical bone, into deep [extrinsic] muscle of tongue [genioglossus, hypoglossus, palataglossus, and styloglossus], maxillary sinus, skin of face).
T4b	T4b Tumour invades masticator space, pterygoid plates, or skull base and/or encases the internal carotid artery.
Oropharynx	
T1	Tumour is 2 cm or less in greatest dimension
T2	Tumour is more than 2 cm but not more than 4 cm in greatest Dimension
T3	Tumour is more than 4 cm in greatest dimension
T4a	Tumour invades the larynx, deep/extrinsic muscle of the tongue, medial pterygoid, hard palate, or mandible.
T4b	Tumour invades the lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, or skull base or encases the carotid artery.
Larynx (supraglottis)	
T1	Tumour is limited to one subsite of the supraglottis, with normal vocal cord mobility.
T2	Tumour invades mucosa of more than one adjacent subsite of the supraglottis or glottis or region outside the supraglottis (e.g., mucosa of base of tongue, vallecula, medial wall of pyriform sinus), without fixation of the larynx.

T3	**Tumour is limited to the larynx with vocal cord fixation and/or invades any of the following: postcricoid area, pre-epiglottic tissues, paraglottic space, and/or minor thyroid cartilage erosion (e.g., inner cortex).**
T4a	Tumour invades through the thyroid cartilage and/or invades tissues beyond the larynx (e.g., trachea, soft tissues of neck, including deep extrinsic muscle of the tongue, strap muscles, thyroid, or esophagus).
T4b	Tumour invades prevertebral space, encases the carotid artery, or invades mediastinal structures.

Larynx (glottis)	
T1	Tumour is limited to the vocal cords(s) (may involve anterior or posterior commissure), with normal mobility.
T1a	Tumour is limited to one vocal cord
T1b	Tumour involves both vocal cords
T2	Tumour extends to the supraglottis and/or subglottis, and/or with impaired vocal cord mobility.
T3	Tumour is limited to the larynx with vocal cord fixation and/or invades paraglottic space, and or minor thyroid cartilage erosion (e.g., inner cortex).
T4a	Tumour invades through the thyroid cartilage and/or invades tissues beyond the larynx (e.g., trachea, soft tissues of the neck, including deep extrinsic muscle of the tongue, strap muscles, thyroid, or esophagus).
T4b	Tumour invades prevertebral space, encases the carotid artery, or invades mediastinal structures.

Larynx (subglottis)	
T1	Tumour is limited to the subglottis.
T2	Tumour extends to the vocal cord(s), with normal or impaired mobility.
T3	Tumour is limited to the larynx, with vocal cord fixation.

T4a	**Tumour invades cricoid or thyroid cartilage and/or invades tissues beyond the larynx (e.g., trachea, soft tissues of neck, including deep extrinsic muscles of the tongue, strap muscles, thyroid, or esophagus).**
T4b	Tumour invades prevertebral space, encases the carotid artery, or invades mediastinal structures.

Hypopharynx	
T1	Tumour is limited to one subsite of the hypopharynx and 2 cm or less in greatest dimension.
T2	Tumour invades more than one subsite of the hypopharynx or an adjacent site, or measures more than 2 cm but not more than 4 cm in greatest dimension without fixation of the hemilarynx.
T3	Tumour is more than 4 cm in greatest dimension or with fixation of the hemilarynx.
T4a	Tumour invades thyroid/cricoid cartilage, hyoid bone, thyroid gland, esophagus, or central compartment soft tissue.
T4b	Tumour invades prevertebral fascia, encases the carotid artery, or involves mediastinal structures.

Salivary gland	
T1	Tumour is 2 cm or less without extraparenchymal extension.
T2	Tumour is greater than 2 cm but not more than 4 cm without extraparenchymal extension.
T3	Tumour is more than 4 cm and/or extraparenchymal extension.
T4a	Tumour invades the skin, mandible, ear canal, and/or facial nerve.
T4b	Tumour invades the skull base and/or pterygoid plates and/or encases the carotid artery.

Neck staging under the TNM staging system for Head and Neck tumours	
Nx	Regional lymph nodes cannot be assessed.
N0	There is no regional nodes metastasis.
N1	Metastasis is in a single ipsilateral lymph node, 3 cm or less in greatest dimension.

N2	**Metastasis is in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension; or metastasis is in multiple ipsilateral lymph nodes, none more that 6 cm in greatest dimension; or metastasis is in bilateral or contralateral lymph nodes, none greater than 6 cm in greatest dimension.**
N2a	Metastasis is in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension.
N2b	Metastasis is in multiple ipsilateral lymph nodes, none more that 6 cm in greatest dimension.
N2c	Metastasis is in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension.
N3	Metastasis is in a lymph node more than 6 cm in greatest dimension.

Distant metastasis(M)	
Mx	Distant metastasis cannot be assessed.
M0	There is no distant metastasis.
M1	There is distant metastasis.

Group staging of Head and neck cancer			
Stage 0	T0	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
Stage IVA	T4a	N0	M0
	T4a	N1	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T4a	N2	M0
Stage IVB	T4b	Any N	M0
	Any T	N3	M0
Stage IVC	Any T	Any N	M1

TNM system often has faced the criticism that it overlooks individual histological features of tumours. It has been suggested that a combined assessment of histological grading along with clinical staging might offer a more precise measure for envisaging the outcome of the neoplasms and determining the best treatment for each patient (Anneroth *et al.*, 1987, Sawair *et al.*, 2003). The histological characteristics of oral cancer may vary widely from site to site within the same tumour, and it is understood that the most valuable prognostic data can be determined from the invasive front of the tumours, where the innate and apparently most aggressive cells reside (Bryne *et al.*, 1989, Bryne *et al.*, 1998). According to 'the dataset for histopathology reporting of mucosal malignancies of the oral cavity' published by the Royal College of Pathologists the following pathological core data items should be included in the histopathology report for oral cancer (Helliwell and Woolgar, 2013):

- Maximum diameter of tumour: The macroscopic diameter in millimetres should be used. If the histological degree is greater than macroscopic extent then the microscopic diameter is used.
- Maximum depth of invasion: For non-ulcerated tumour the maximum depth of invasion underneath the luminal aspect of surface should be documented. For ulcerated tumour the reconstructed surface should be used. A more detailed nature of the tissues invaded (mucosa, muscle etc.) should be commented. Depth of invasion has a good evidence for the prognostic value in oral cancer.

- Histological type of carcinoma: Subtypes of squamous carcinoma, such as basaloid, papillary, verrucous, adenosquamous etc. should be identified and listed in the dataset.
- Degree of differentiation (grade): Tumour is graded according to the degree of resemblance of the carcinoma to the normal epithelium and the most aggressive area is graded as poorly, moderately and well differentiated. Grading is useful for prognostication.
- Pattern of invasion: Two groups for the pattern of invasion are identified for the purposes of prognostication: 'carcinomas composed of broad cohesive sheets of cells or strands of cells >15 cells across' and 'carcinomas composed of narrow strands, non-cohesive small groups of single cells'.
- Distance from invasive carcinoma to surgical margins: Distance for both mucosal and deep margins should be measured histologically. >5mm is clear, 1-5mm is close and <1mm is involved, from a surgical point of view.
- Vascular invasion: If vascular invasion is an obvious feature on medium magnification examination of the tumour, the presence or absence of it should be mentioned.
- Nerve invasion: Perineural invasion in front of the invasive front of the carcinoma should be documented, regardless of the size of the nerve. Nerve invasion predicts more aggressive disease.
- Bone invasion: The presence or absence of carcinoma at the bone margins should be listed, if bone invasion is present.

- Severe dysplasia/carcinoma *in-situ*: The presence of moderate or severe dysplasia next to to the primary carcinoma and inside 5mm of the resection margins should be noted. Severe dysplasia and carcinoma *in-situ* often used interchangeably and linked with a high risk of progression to carcinoma.

1.4 Epidemiology of Head and Neck cancer

The world cancer report 2014 published that there were around 15 million new cancer cases, 8.5 million cancer deaths and 33 million people living with cancer within 5 years of diagnosis in 2012 worldwide. 58% of new cancer cases, 66% of the cancer deaths and 49% of the 5-year prevalent cancer cases appeared in the less developed countries (Stewart and Wild, 2014). In 2014, the International Agency for Research on Cancer (IARC) estimated that there are approximately 630,000 new cases of head and neck cancer and 300,000 deaths every year worldwide, which makes it the 6th most prevalent cancer in the world. The most common sites of head and neck cancer are the oral cavity with 400,000 cases a year, the larynx with 160, 000 cases a year and the pharynx with 65,000 a year (Stewart and Wild, 2014).

There is an approximately 20-fold geographical variation in the incidence of this cancer. South and Southeast Asia (e.g. India, Bangladesh, Sri Lanka, Taiwan), parts of Latin America and the Caribbean (e.g. Brazil, Puerto Rico, Uruguay), parts of

Western (e.g., France, Eastern Europe: Hungary, Slovakia, Slovenia) and in Pacific regions (e.g. Papua New Guinea, Melanesia) are the areas characterised by high incidence rates for oral cancer. This cancer is the most common cancer in men in high-risk countries such as India, Bangladesh, Pakistan and Sri Lanka and may represent up to 25% of all new cancer cases (Warnakulasuriya, 2009). WHO projections estimated mortality statistics from oral and oropharyngeal cancer in 2008 to be 371, 000 worldwide. Because there is a predicted rise in mortality in Southeast Asia (182,000 in 2008 to 324,000 in 2030) this is projected to increase to 595,000 by 2030 worldwide (Mathers and Loncar, 2006, Mehanna *et al.*, 2010).

A recent study however revealed that, rates of oral cavity cancer have decreased in Canada and the US, remained stable or decreased in a number of Asian countries and increased in a number of European countries. Oropharyngeal cancer rates also decreased for men and women in China (Hong Kong) and India (Mumbai) and rates rise among men and women in some eastern (Belarus, Czech Republic) and in some northern European countries (the UK, Sweden, Norway, Finland) as well as some Asian countries (Japan, India[Chennai]). The difference in trends in incidence rates among countries by head and neck cancer sub-site and gender probably indicate the differences in the prevalence of known risk factors (Simard *et al.*, 2014).

In the UK, around 6,500 people were diagnosed with oral cancer in 2010 that equates to 18 people every day. Among them, 4,300 were men and 2,200 were women. The incidence rate of oral cancer has increased by a third in the last decade. The most common age group is 65 and over for oral cancer cases in the UK. On the other hand,

around 2,400 people were diagnosed with laryngeal cancer in 2011, more than 6 people a day. 1,900 were men and 400 were women were diagnosed with laryngeal cancer, i.e. it is four times more common in men that women.

In 2011, around 2,100 people died of oral cancer (more than five people a day) and 800 people died of laryngeal cancer in the UK. Around two-thirds of oral cancer deaths and 8 in 10 laryngeal cancer deaths were in men. The mortality rates of oral cancer have remained stable since 1970, whereas the laryngeal cancer mortality rate have fallen by around a fifth in the last decade (CRUK, 2014a, b).

Scotland has the highest occurrence of oral cancer in the UK (Table 3)(CRUK, 2014c) and this incidence is rising alarmingly (Robinson and Macfarlane, 2003, Mendez, 2007). Taken together, oral and laryngeal cancer incidence occurs in 2.7% of all malignancies which rank head and neck cancer the 10th most prevalent cancer in the UK (CRUK, 2014c).

Table 3 UK Head and Neck cancer incidence rate (2011) by country

HNSCC by site	**Incidence rate per 100,000**				
	England	Wales	Scotland	Northern Ireland	UK
Oral	8.6	10.1	11.8	9.3	9.0
Larynx	2.7	3	4.2	4.3	2.9

Notes: Rates are European age-standardised and given per 100,000 populations (CRUK, 2014c).

1.5 Aetiology and risk factors

A number of risk factors have been characterised as being associated with oral cancer with substantial evidence (Ram *et al.*, 2011). Not all individuals exposed to these risk factors develop oral cancer; on the contrary, oral cancer may also develop without exposure to these risk factors. This observation proposes that additional causes, for example genetic susceptibility, may also play a part in the variable individual susceptibility to oral cancer (Mehrotra and Yadav, 2006). Cancer is the eventual consequence of the transformation of normal cells to mutated cells with uncontrolled growth by enhancing factors, DNA-reactive and genotoxic carcinogens. Thus, cancer is the product of interaction of environmental exposures and genetic factors which progresses over a long time and goes through many steps (Taghavi and Yazdi, 2007). Some well characterised risk factors are described below:

1.5.1 Tobacco use

There is plenty of evidence to suggest that tobacco in different forms, including smoking and smokeless have a carcinogenic impact in the oral cavity. Smoking is the commonest form of tobacco use and the most important risk factor for oral cancer. Smoking of tobacco as factory-made cigarettes, cigars and cheroots and loose tobacco in pipes or hand-made cigarettes is familiar to all (Johnson, 2001). The risk is higher for heavy smokers, long-term smokers and smokers of black or high tar cigarettes. Cigar and pipe smoking also pose a risk. (Kjaerheim *et al.*, 1998). Smoking

of bidis (small filter-less cigarettes common in India and Bangladesh) seems to have a higher risk for cancer of the hypo-pharynx and larynx than smoking of the Western type cigarettes (Sapkota *et al.*, 2007). Hookah or chillum, a clay pipe used to keep the burning tobacco are other common forms of smoking in some countries of Asia including India (Ram *et al.*, 2011). Among men in industrialised countries, smoking is estimated to be the cause of 40-45 percent of all cancer deaths, over 85 percent of oral cancer deaths, in those aged thirty-five to sixty-nine years (Boyle and Levin, 2008). It is estimated that smoking tobacco causes around 70% of oral and pharyngeal cancers in men, and around 55% in women in the UK (Parkin, 2011a). Smokeless tobacco is consumed orally or nasally, without burning the product. Oral smokeless products are placed in the mouth, cheek or lip and are sucked (dipped) or chewed. Chewing tobacco can be classified as loose leaf, plug and twist. Snuff is a common term for finely cut or powdered, flavoured tobacco, which can be prepared as moist or dry snuff. Tobacco pastes or powders are also used orally and rub on to the gums or teeth. Dry snuff can also be inhaled through the nasal passages (Boffetta *et al.*, 2008). In developing countries smokeless tobacco is mostly used mixed with other constituents (Table 4) (Figure 1.23) (Johnson, 2001).

Table 4 Some conventional forms of oral smokeless tobacco

Habit	**Ingredients**	**Population**
Pan/Paan/betel quid	Areca nut, betel leaf, slaked lime, catechu, condiments,	Indian subcontinent, Southeast Asia, Papua New Guinea, part

	with or without tobacco	of South America
Khaini	Tobacco and lime	Bihar (India)
Mishri	Burned tobacco	Maharashtra (India)
Zarda	Boiled tobacco	India, Bangladesh and Arab countries
Gudkha/Gudakhu	Tobacco and molasses	Central India, Pakistan
Mawa	Tobacco, lime and areca	India
Nass	Tobacco, ash, cotton or seasame oil	Central Asia, Iran, Afganistan, Pakistan
Naswar/niswar	Tobacco, lime, indigo, cardamom, oil, menthol, etc.	Central Asia, Iran, Afganistan, Pakistan
Shammah	Tobacco, ash and lime	Saudi Arabia
Toombak	Tobacco and Sodium bicarbonate	Sudan

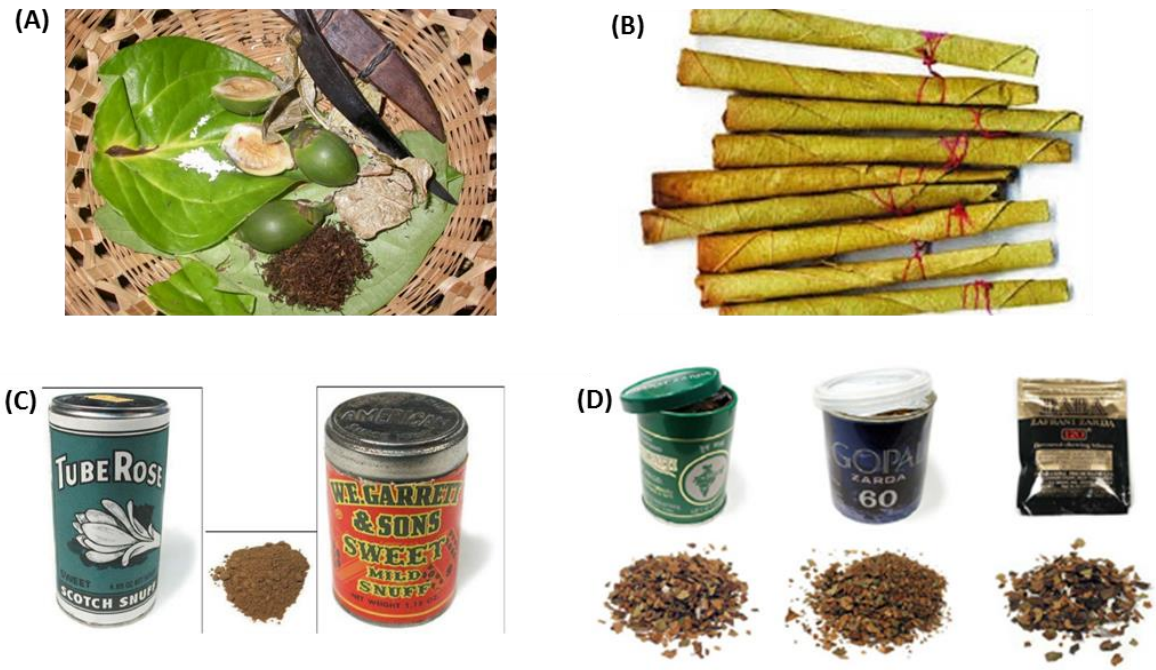


Figure 1.24 Some common forms of smokeless tobacco product.
(A) Betel quid with tobacco, (B) Bidi, (C) Dry snuff and (D) Zarda.

IARC categorises smokeless tobacco as a cause of oral cavity cancer. Oral cancer risk varies by geographical location, possibly due to variances in the composition and format (e.g. chewing or inhaling nasally) of smokeless tobacco (IARC, 2014). In India and Sudan, over 50% of oral cancers are attributable to smokeless tobacco products used in these countries, so are about 4% of oral cancers in US men and 20% of oesophageal cancers in Swedish men (Boffetta *et al.*, 2008).

Tobacco is the most common source of carcinogens to human, including polycyclic aromatic hydrocarbons (i.e. benzo[a] pyrene) and tobacco specific nitrosamines (i.e. NNK). It introduces a linear dose-response carcinogenic effect in which duration is more significant than the intensity of exposure. The most important carcinogenic effect of tobacco resides in the tar fraction, which comprises a composite

mixture of interacting cancer initiators, promoters, and co-carcinogens (Sturgis *et al.*, 2004). The chronic exposure of this mixture to the airway epithelial cells, through continued smoking, can cause molecular lesions which, in the presence of reduced metabolic detoxification, can weaken repair capability, overwhelming cellular defences and lead to oral cancer (Boffetta *et al.*, 2008, Boyle and Levin, 2008) .

1.5.2 Alcohol

Alcohol is also an important promoter of carcinogenesis and contributes in at least 75% of Head and Neck cancer (Blot *et al.*, 1988). WCRF/AIRC and IARC classify alcohol as a risk factor of oral cavity, tonsil and pharyngeal cancer (Wiseman, 2008, IARC, 2014). It is estimated that alcohol drinking causes around 37% of oral and pharyngeal cancers in men and around 17% in women in the UK (Parkin, 2011b). A meta-analysis showed that regular alcohol drinkers have around 2.5 times the risk of oral and pharyngeal cancer compared with non-drinkers and occasional drinkers. The same study also showed that heavy drinkers who drink around 6 units or more per day increase the risk of oral and pharyngeal cancer more than five- fold (Turati *et al.*, 2013). It has been suggested that alcohol may have additive effects and it facilitates the entry of carcinogens into the exposed cells, altering the metabolism of mucosal cells (McCoy, 1978). The meta-analysis of Turati (2013) has also revealed that alcohol increases the risk of oral and pharyngeal cancer in current smokers rather than never/former smokers. Heavy drinking smokers have an almost 7-fold higher risk than heavy-drinking non-smokers (Turati *et al.*, 2013). Alcohol in

conjunction with smokeless tobacco also increases oral cancer risk (Znaor *et al.*, 2003, Lin *et al.*, 2011).

1.5.3 Human Papilloma Virus (HPV)

IARC classifies HPV-16 and HPV-18 as a cause for oral cavity, tonsil and pharynx cancer and probable cause of oral cancer, respectively (IARC, 2014). It is estimated that around 8% of oral cavity cancers and 14% of oropharyngeal cancer is caused by HPV infection in the UK (Parkin, 2011c). A meta-analysis shows that around 40% of oropharyngeal cancer cases in Europe are HPV-positive. Data obtained from 2005 onwards suggest that the HPV prevalence rate in oropharyngeal cancer has increased and reached over 70%. For oral cavity, hypopharynx and larynx cancer (non-oropharyngeal head & neck cancer), HPV prevalence in European cases has not significantly increased over time and its rate is 24% (Mehanna *et al.*, 2013). Sexual activity is the main cause of HPV infection. A pooled-analysis showed that multiple past sex partners, especially oral sex partners and sexual activity at a younger age have increased the risk of oropharyngeal, tongue and base of tongue cancer (Heck *et al.*, 2010).

1.5.4 Epstein-Barr Virus (EBV)

IARC classifies EBV infection as a main cause of nasopharyngeal cancer. It is estimated that 90% of nasopharyngeal cases in the UK involved EBV infection equating 6% of overall oral cavity and pharynx cancer (Parkin, 2011c).

1.5.5 HIV/AIDS

Meta-analyses have shown that HIV positive patients have doubled the risk of oral cavity, oropharyngeal and pharynx cancer compared to the general population (Grulich *et al.*, 2007, Shiels *et al.*, 2009). There is also a positive relationship between HIV and HPV infection (Houlihan *et al.*, 2012).

1.5.6 Immunosuppression

A meta-analysis and several large cohort studies have shown that people who have undergone organ transplants have 2 to 5 times the risk of oral cavity and pharynx cancer compared to general population (Grulich *et al.*, 2007, Engels *et al.*, 2011, Krynitz *et al.*, 2013). The risk of lip cancer increases by 17-46 times in transplant recipients (Grulich *et al.*, 2007, Engels *et al.*, 2011, Krynitz *et al.*, 2013). Kidney, heart and lung recipients are particularly prone to lip cancer and which is increased with current immunosuppressant medication and smoking. The possible explanations for increased risk of lip cancer include continued HPV infection and increased sensitivity to UV radiation as a function of immunosuppressant medication (van Leeuwen *et al.*, 2009, Krynitz *et al.*, 2013).

1.5.7 Radiation

According to IARC solar radiation is the probable cause of lip cancer. IARC also classifies X-Ray and Gamma radiation as the causes of salivary gland cancer and radioiodine as a probable cause (IARC, 2014). Most modern evidence of the effect of

ionising radiation comes from studies of people exposed to diagnostic or therapeutic medical radiation, whereas early evidence came from atomic bomb survivors (Saku *et al.*, 1997). Studies have shown that survivors of childhood cancer and Hodgkin Lymphoma possess 17 and 39-fold higher risk of salivary gland cancer, respectively due to the use of radiation to eradicate the primary tumour (Boukheris *et al.*, 2008, Boukheris *et al.*, 2013). Thyroid cancer survivors have between 3 to 11 times the higher risks of salivary gland cancer, attributable to radioiodine treatment (Sawka *et al.*, 2009, Iyer *et al.*, 2011).

1.5.8 Oral condition and medication

Oral epithelial dysplasia and oral leucoplakia has the potential to undergo malignant transformation to OSCC (Nankivell *et al.*, 2012). The transformation rate published in literature varied from 5 to 36 % (Silverman *et al.*, 1984, Hsue *et al.*, 2007). A meta-analysis showed that around 12% of these conditions will transform into cancer (Mehanna *et al.*, 2009). Another meta-analysis has shown that patients with gum disease have 2.6-fold higher risk of head and neck cancer (Zeng *et al.*, 2013a). A meta-analysis also showed that people who lose 6 or more teeth have at least a 60% increased risk of head and neck cancer and the risk is positively correlated with the number of teeth lost (Zeng *et al.*, 2013b). White people who used hydrochlorothiazide for five years or more, which has a photosensitising effect had a 4-fold increased risk of lip cancer compared to non-users, a US cohort study showed (Friedman *et al.*, 2012).

1.5.9 Familial and genetic predisposition

Previous cancer can precede increased risk of oral cancer due to shared aetiological factors (HPV, smoking etc.) and the effect of radiotherapy for the first cancer. A pooled-analysis showed that people with previous head and neck cancer have 12-16 times increased risk of subsequent head and neck cancer (Bosetti *et al.*, 2011). Survivors of oesophageal squamous cell carcinoma have almost 7-times increased risk of oral cavity and pharynx cancers (Chuang *et al.*, 2008). Lung cancer survivors have 1.5 to 5.7 times increased risk of developing head and neck cancer varying by sex and lung cancer histology (Chuang *et al.*, 2010). Previous cervical cancer patients also have increased risk of mouth and pharynx cancer (Chaturvedi *et al.*, 2009).
A pooled-analysis showed that a family history of head and neck cancer particularly in siblings have almost 70% increased risk of head and neck cancer. Individuals who smoke tobacco and drink alcohol with a family history of head and neck cancer have more than a 7-times increased risk (Negri *et al.*, 2009).

1.5.10 Occupational hazards

It is estimated that in Great Britain in 2004 occupational exposure to formaldehyde and wood dust caused around 11% of nasopharyngeal cancer in men and around 2% in women (Slack *et al.*, 2012). A US cohort study showed that embalmers and funeral directors who were heavily exposed to formaldehyde have twice the risk of dying from nasopharyngeal cancer (Hauptmann *et al.*, 2004). A pooled- analysis also showed that furniture and plywood workers have around a 140% increased risk of

nasopharyngeal cancer (Demers *et al.*, 1995). A meta-analysis also revealed that people exposed to asbestos and printing processes (polycyclic aromatic hydrocarbons and mineral oils) have a 25% and 14% increased risk of oral and pharyngeal cancer, respectively (Paget-Bailly *et al.*, 2012).

1.5.11 Dietary habit

IARC classifies Chinese-style salted fish as a cause of nasopharyngeal cancer (IARC, 2014). There is a higher amount of nitrosamines found in the Chinese-style salted fish than other type of salted fish. A case-control study showed that people who intake the highest amount of Chinese-style salted fish in childhood exhibited an increased risk, 2.5 times the risk of nasopharyngeal cancer in adulthood (Jia *et al.*, 2010). However, another study found that after controlling the tobacco use and vegetable intake, salted fish intake was not associated with the risk of nasopharyngeal cancer (Lau *et al.*, 2013). Drinking hot maté, a traditional Latin American infused drink is found to be a risk factor for pharyngeal cancer (Wiseman, 2008, IARC, 2014). A meta-analysis from case-control studies from Latin America showed that maté users have around twice the risk of oropharyngeal cancer that low or never-users, although it remains unclear whether this is due to the hot temperature of the drink or any carcinogenic compounds present in the drink (Dasanayake *et al.*, 2010).

1.5.12 Socio-economic factors

The complex association between the risk of head and neck cancer and socio-economic classes were explored in a study in Scotland. People living in the most deprived areas and those who were unemployed had a significantly higher risk of head and neck cancer than those with high level of educational accomplishment, however smoking dominated the overall profile of risk (Conway *et al.*, 2010).

The oral cancer risk factors evaluated by IARC and WCRF/AICR are summarised in the following table.

Table 5 IARC and WCRF/AICR evaluations of oral cancer risk factors

Increases risk	**May increase risk**
• Alcohol (oral cavity, tonsil, pharynx) • Betel quid with tobacco (oral cavity, tonsil, pharynx) • Betel quid without tobacco (oral cavity) • HPV-16 (oral cavity, tonsil, pharynx) • Smokeless tobacco (oral cavity) • Tobacco smoking (oral cavity, tonsil, pharynx, nasopharynx) • X-radiation, gamma-radiation (salivary gland)	• Hydrochlorothiazide (lip) • Solar radiation (lip) • HPV-18 (oral cavity) • Radioiodines, including iodine-131(salivary gland) • Asbestos (pharynx) • Hot Maté drinking (pharynx) • Printing processes (pharynx) • Passive smoking (pharynx)

• EBV (nasopharynx) • Formaldehyde (nasopharynx) • Chinese-style salted fish (nasopharynx) • Wood dust (nasopharynx)	

Adapted from CR-UK website (CR-UK, 2013)

1.6 General overview of signal transduction

Communication between cells, tissues and organs is controlled by internal and external signals that either stimulate or inhibit biological processes. One of the fundamental issues in current cell biological research is the mechanism by which cells identify and react to these signals. Signal transduction is a series of collective reactions by which the biological effects of different regulatory molecules are transmitted from the cell membrane to intracellular targets such as the nucleus, mitochondria and the endoplasmic reticulum. This transmission of signals from membrane to internal targets leads to a series of molecular events that translate into the eventual biological effects (Mohan, 2009).

Cells continuously communicate with each other. This communication takes place through both electrical and chemical signals.

Electrical signals pass directly from one cell to its neighbour through the gap junction and this is very fast. This type of signal can be found mainly in the brain and heart. The major form of information transfer between cells is the

communication through chemical signals. When the cell releases the chemical stimulus such as a growth factor, it then changes the action of target cells. Target cells have receptors able to sense the incoming signal and transmit the information to the appropriate internal cell signalling pathway which results in a change in cellular activity (Berridge, 2009, Powar, 2010) (Figure 1.24).

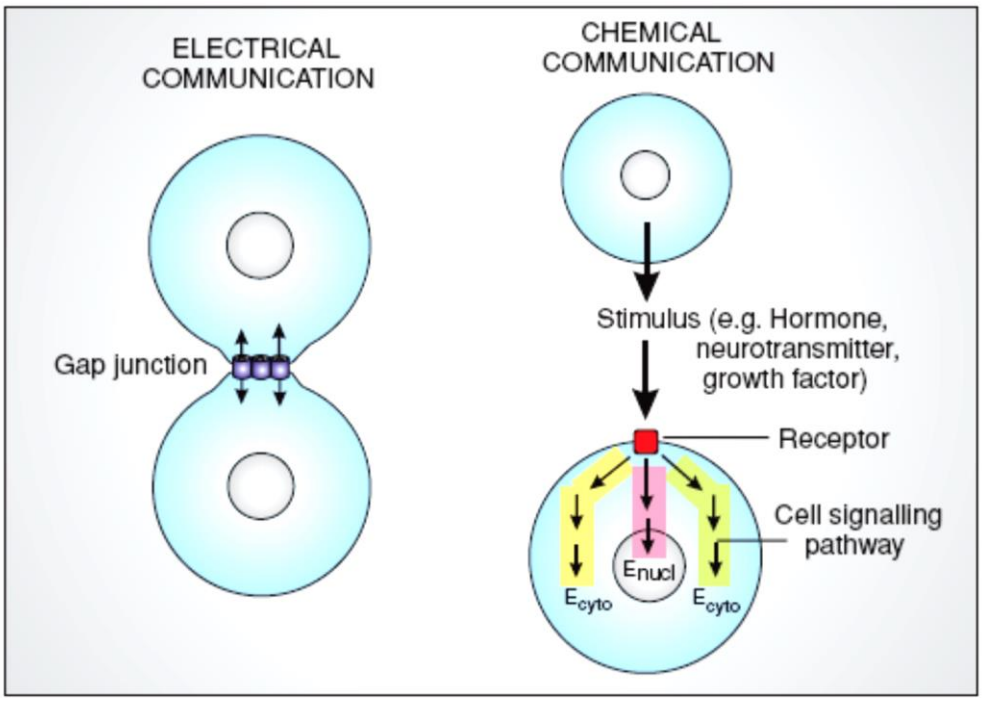


Figure 1.25 Cell communication through electrical and chemical signalling mechanisms.

Cells that are linked through the low-resistance gap junctions can communicate quickly with each other by transmitting electrical current or through the circulation of low-molecular-mass second messengers such as cyclic AMP and inositol 1,4,5-trisphosphate (InsP3). In the case of chemical communication, the cell discharges a stimulus, which is dispersed to a target cell that has receptors to sense the stimulus and to pass the information along a range of cell signalling pathways to trigger effectors either in the nucleus (Enucl) or cytoplasm (Ecyto) (Berridge, 2009).

The fundamental theory of a cellular transduction pathway, therefore, concerns the mechanisms liable for accepting the external signal and transmitting it through internal cell signalling pathways to trigger the sensor and effector mechanisms that lead to transforming in cellular activities (Figure 1.25). This pathway transduces signals to different parts of the cells, but most frequently to the genes. The term 'transduce' is employed here in the sense of to send or carry.

Cell communication by extracellular signals typically contains the following series of steps:

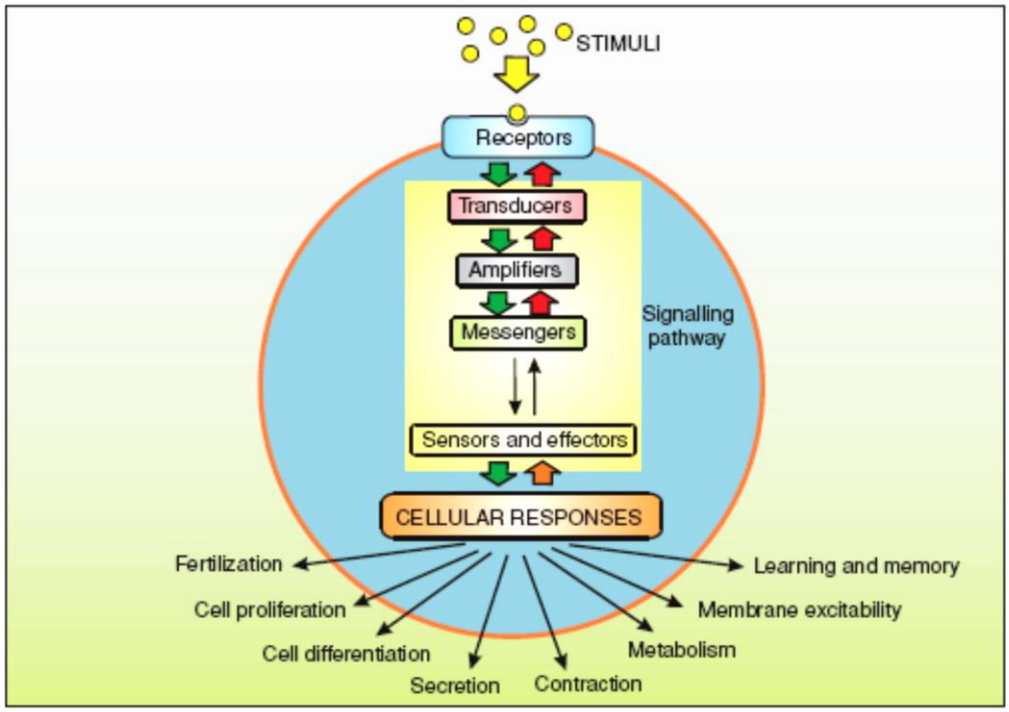


Figure 1.26 The fundamental theory of a cellular transduction pathway.
Stimuli are bound to cell-surface receptors which pass the information through intracellular signalling pathways that can have a number of different mechanisms. They typically start with the activation of transducers that employ amplifiers to produce internal messengers that either operate locally or can disperse throughout the cell. These messengers then employ sensors that are attached to the effectors that

are liable to trigger the cellular responses. The green and red arrows point out that cell signalling is a dynamic process comprised of ON mechanisms (green arrows) through which information flows down the pathway, opposite to the OFF mechanisms (red arrows) that turn off the diverse steps of the signalling pathway (Berridge, 2009).

Synthesis of the signalling molecules or ligands: Cells communicate with each other by extracellular physical or chemical signals in multicellular organisms. Signalling cells synthesise the chemical signals. Proteins and peptides, hormones and neurotransmitters are the predominant forms of chemical signals. Heat and lights are the physical signals and are not produced by the signalling cells (Powar, 2010).

Release of the ligands: These signalling molecules are then secreted by the cells and generate a specific response in target cells (Powar, 2010).

Transport of the ligands: Transfer of the signalling molecules to the target cells occurs over varying distances. Signalling molecules such as hormones, acts on target cells distant from the cell they are produced by and this is termed as endocrine signalling. In paracrine signalling, ligands act locally on nearby cells. In autocrine signalling the molecules act on the same cells that synthesise them. Juxtacrine refers to direct cell to cell contact dependent signalling (Berridge, 2009, Powar, 2010).

Signal detection by receptors: Ligands interact with signalling pathways by specifically binding with protein receptors. Lipid soluble amphiphilic ligands diffuse across the cell plasma membrane and bind with intracellular receptors. In contrast,

low lipid soluble ligands cannot cross the plasma membrane and therefore, bind with the membrane receptors. Upon binding of ligands, receptors may undergo conformational change in their structure and transfer the signal into the cell. Intrinsic or associated catalytic activity of the receptors may modify intracellular target proteins (Lauffenburger and Linderman, 1993, Lodish *et al.*, 2007).

Receptor-Signal complex: Alteration in the cells triggered by receptor-signal complex may be in the form of metabolism, development or function. Chains of intercommunicating proteins of signalling pathways convey the signal in a stepwise manner. The signalling pathway may end in the nucleus with DNA-binding proteins that repress or trigger replication or transcription (Nelson, 2008, Powar, 2010).

Signal termination: Termination of the signals can occur by removal of the ligands, down-regulation or desensitisation of the receptors (Dennis and Bradshaw, 2011).

1.6.1 Signalling molecules or ligands

Ligands are a chemically varied group of molecules that transmit signals between the cells by binding to plasma membrane receptors. Ligands are primarily classified into three categories based upon the distance they travel.

I. Hormones: They are secreted by specific endocrine cells into the blood stream and act on the target cells remote from the site of production. Based on their solubility and their receptor location in the cells they are grouped into three categories.

i. *Lipophilic hormones with intracellular receptors*: They are small lipid soluble molecules that can cross the plasma membrane and bind with intracellular receptors either in the cytoplasm or in the nucleus. Examples include steroid hormones like oestrogen and progesterone, Vitamin D and thyroxine (Berridge, 2009).

ii. *Lipophilic hormones bind to plasma membrane receptors:* This type of hormone such as the Prostaglandins can act both as autocrine and paracrine signalling molecules (Berridge, 2009, Powar, 2010).

iii. *Hydrophilic hormones bind to plasma membrane receptors:* These are water soluble hormones that cannot diffuse across the lipid bilayer of the plasma membrane and so therefore bind with the plasma membrane receptors. Examples includes peptide hormones (insulin and glucagon), and biogenic amines (adrenaline and histamine) (Marks *et al.*, 2008, Gomperts *et al.*, 2009).

II. Neurotransmitter: They are released into the synapses of the nerve cells and travel a very short distance from pre-synaptic to post-synaptic neurons. So therefore they act as paracrine signalling molecules (Dennis and Bradshaw, 2011).

III. Local mediators: This type of signalling molecules are secreted by various types of cells and act on close by neighbouring cells. Examples of local mediators include protein growth factors, amino acid derivatives, lymphokines and fatty acid derivatives (Nelson, 2008).

1.6.2. Signalling receptors

Ligand binding to the receptors causes conformational change of the receptors that initiates a series of reactions and ultimately causes cellular response. Receptors are protein molecules that have binding specificity for a specific ligand. Different cell types may have different sets of receptors for the same type of ligand inducing different cellular responses. In contrast, different cell types may have the same receptors and upon binding of the same ligand can cause different cellular activity. Based on their location, receptors are primarily classified by two types which are then sub-divided into different categories (Table 6) (Lauffenburger and Linderman, 1993, Gomperts *et al.*, 2009).

Table 6 Different types of signalling receptors

I. Cell Surface Signalling Receptors
IA. Ion Channel linked Receptors
IA 1. Ligand-gated ion channel
IA 2. Voltage-gated ion channel

IA 3. Second-messenger gated ion channel

IB. Metabotropic Receptors

IB 1. G protein-coupled Receptors (GPCRs)

IB 2. Enzyme-linked Cell surface receptors

IB2a. Receptors with Intrinsic enzymatic activity

IB2a i. Receptor Tyrosine Kinases (RTKs)

IB2a ii. Receptor Serine/Threonine Kinases

IB2a iii. Receptor guanylyl cyclises (RGCs)

IB2a iv. Receptor-like protein tyrosine phoshphatases (PTPs)

IB 2b. Receptors with associated enzymatic activity

IB2b i. Receptors with associated tyrosine kinase activity

IB2b ii. Receptors with associated histidine kinase activity

II. Intracellular Signalling Receptors

IIA. Cytosolic Receptors

IIB. Nuclear Receptors

I. Cell surface signalling receptors

Most of the ligands are hydrophilic in nature and so they cannot cross the lipid bilayer of the plasma membrane. These ligands interact with transmembrane or membrane spanning or membrane associated receptors. Upon binding, ligands can change the structure of the receptors which initiates the signal transduction pathway. Ion channel-linked and metabotropic receptors are two broad classes of the

cell surface receptors which can activates a series of enzymes in the ligand-bound state (Nelson, 2008, Powar, 2010).

IA. Ion channel-linked or Ionotropic receptors

Ion channel linked receptors have inherent channels which are closed in the default state and unlock in response to a particular signal. This channel is controlled by closing and opening which allows or prevents ions or small molecules in or out of the cell. These channels can be voltage gated, ligand gated or second-messenger gated (Lauffenburger and Linderman, 1993, Powar, 2010).

IA 1. Ligand-gated ion channel

This type of ion channels open in response to a particular type of ligand e.g. neurotransmitter which may be excitatory and inhibitory. Excitatory neurotransmitters include glutamate, acetylcholine and ATP. Inhibitory neurotransmitters include GABA (Dennis and Bradshaw, 2011).

IA 2. Voltage-gated ion channel

This type of ion channel opens in response to electrical changes related to an action potential in neurons (Hancock, 2010).

IA 3. Second-messenger gated ion channel

This type of channel receptors acts in response to second messengers such as cAMP and Ca^{2+} in the cell (Berridge, 2009).

IB. Metabotropic Receptors

Metabotropic receptors can activate a particular series of enzymes in the ligand bound state. They can be GPCR and enzyme-coupled receptors (Berridge, 2009, Sitaramayya, 2009).

IB 1. G protein-coupled receptor

These types of receptors are integral membrane proteins composed of a single polypeptide which forms seven transmembrane domain structures. The ligand binding site is in an extracellular N-terminal region, the seven transmembrane domain structure is a central hydrophobic region which forms a helical wheel structure and the cytosolic C terminal is associated with a heterotrimeric G protein. GPCRs activate a GTP binding protein called G-protein, which then activates enzymes in the cytosol or loosely associated with the inner side of the plasma membrane. G-proteins act as intermediates in the signal transduction pathway which can determine whether a signal will be stimulatory or inhibitory (Gomperts *et al.*, 2009, Powar, 2010).

IB 2. Enzyme-linked cell surface receptors

These types of receptors are the second major type of metabotropic receptors. In contrast to GPCRs, they have a single transmembrane domain structure. The N-terminal region is in the extracellular part and is for ligand binding. The cytosolic C –terminal region either has intrinsic enzymatic activity or is directly associated with enzymes. These types of receptors are collectively called growth-factor receptors, because the ligands which bind to these receptors are responsible for cellular

growth, differentiation and proliferation. The responses mediated by these receptors normally are slow because a number of intracellular signalling steps are involved. However, these receptors also mediate a direct and rapid effect on the cellular cytoskeleton which control cell movement and shape (Lauffenburger and Linderman, 1993, Gomperts *et al.*, 2009, Powar, 2010).

There are six types of enzyme-linked cell surface receptors among which four of them have intrinsic enzymatic activity and two have associated enzymatic activity.

IB2a. Receptors with enzymatic activity

i. Receptors Tyrosine Kinases (RTKs): RTKs phosphorylate specific tyrosine residues on a restricted set of intracellular signalling proteins (Lauffenburger and Linderman, 1993, Powar, 2010).

ii. Receptor Guanylyl Cyclases (RGCs): RGCs catalyse cGMP production in the cytosol(Lauffenburger and Linderman, 1993).

iii. Receptor Serine/Threonine kinases: This type of receptors phosphorylate specific serine/threonine residues on related gene regulatory proteins (Lauffenburger and Linderman, 1993, Berridge, 2009).

iv. Receptor like protein tyrosine phoshphatases (PTPs): They can dephosphorylate phosphotyrosine (Powar, 2010).

IB2b. Receptors with associated enzymatic activity

i. Receptors with associated tyrosine kinase activity are linked with intracellular proteins containing tyrosine kinase activity. For example, JAK-STAT pathway (Lauffenburger and Linderman, 1993, Powar, 2010).

ii. Receptors with associated histidine kinase activity autophosphorylate histidine in the two compartment signalling pathway (Berridge, 2009, Powar, 2010).

1.6.3. Transducers and amplifiers

They are regarded as together because their activities are closely connected and every so often, the two functions exist in the same molecule. They are linked with the receptors, accept information coming in from the outside and transform it into the internal messengers. One of the typical examples of information transduction is GPCRs that use heterotrimeric G proteins to transmit information to amplifiers such as Adenylyl cyclase or phospholipase C (PLC).The monomeric G-proteins like SoS (Son of Sevenless) function as transducers for the tyrosine kinase-linked receptors. The ion channel receptors are receptors where a single protein combines the role of receptor, transducer and amplifier. Different transducers and amplifiers generate different second messengers that transmit information to the internal sensors and effectors (Berridge, 2009, Powar, 2010, Dennis and Bradshaw, 2011).

1.6.4. Intracellular messenger

Intracellular messengers are the agents that transmit the information within the cell. They can take many different forms.

a. One of the major intracellular messengers is Ca^{2+} that carries information for the Ca^{2+} signalling pathway (Berridge, 2009, Hancock, 2010).
b. cAMP that transmit information for the cAMP signalling pathway (Berridge, 2009, Dennis and Bradshaw, 2011).
c. Ca^{2+} mobilising messengers:
    - Inositol 1, 4, 5 tri phosphate (IP3) acts as a messenger connecting receptors to the Ca^{2+} signalling pathway (Lodish *et al.*, 2007, Dennis and Bradshaw, 2011).
    - Cyclic ADP ribose that discharges Ca^{2+} from internal stores (Hancock, 2010, Powar, 2010).
d. Lipid messengers:
    - DAG (Diacyl Glycerol) connects receptor activation to protein kinase C and protein phosphorylation of the phosphoinositide pathway (Berridge, 2009).
    - PI3,4,5P3 acts as a lipid messenger in the PI3-kinase pathway (Lodish *et al.*, 2007, Berridge, 2009).

- PI4,5P2 acts as a membrane messenger to trigger various cellular processes (Berridge, 2009, Dennis and Bradshaw, 2011).
- Phosphatidic acid (PA) is a messenger for Phospholipase D signalling pathway (Berridge, 2009).

e. Protein kinase messengers: Particular protein kinases can transmit information to diverse area of the cells.
    - ERk1/2 and JNK active kinases generated by MAPK signalling pathway can transmit information into the cell and very often into the nucleus (Berridge, 2009, Dennis and Bradshaw, 2011).

f. Some transcription factors activated at the membrane or in the cytoplasm can act as messengers that transmit information into the nucleus.
    - NFkB and STATs can transmit information from the cell surface to the nucleus (Berridge, 2009, Dennis and Bradshaw, 2011).
    - Smads carry signals from the TGFβ- superfamily receptors into the nucleus (Berridge, 2009, Hancock, 2010).
    - β-catenin acts as a messenger in the canonical Wnt pathway (Marks *et al.*, 2008, Berridge, 2009).

1.6.5. Sensors and effectors

Intracellular messengers can carry information to the sensors and effectors that are responsible for the final task of the cell signalling pathways to trigger a whole host of cellular processes. For example, Ca^{2+} binding proteins can detect increasing levels of Ca^{2+} and convey this signal to diverse effector molecules which control the processes of contraction and secretion. Some of the effectors are somewhat simple consisting of a single downstream effector system, but some of them are more complicated being made up of various components such as those that limit the processes of gene transcription, actin remodelling, phagocytosis and exocytosis. The activation of these sensors and effectors terminates the passage of the information down the cell signalling pathway (Marks *et al.*, 2008, Berridge, 2009, Powar, 2010, Dennis and Bradshaw, 2011).

1.7 Growth factors in HNSCC

Growth factors and their specific cell surface receptors harbouring a tyrosine kinase activity offer us profound knowledge in identifying the mechanism of development, homeostasis and diseases (Higashiyama *et al.*, 2008). Various growth factors and their signalling pathways give us remarkable advantages in developing intriguing molecular targeted therapy in various cancers including HNSCC.

1.7.1 The Epidermal Growth Factor (EGF) family

The EGF family of growth factors are characterised by the existence of an EGF-like domain which is composed of three disulphide-bonded intramolecular groups conferring binding specificity and supplementary structural motifs such as immunoglobulin-like motifs, glycosylation sites and heparin-binding sites (Normanno *et al.*, 2005). The EGF family includes 13 members: EGF, TGF-α (transforming growth factor-α). Amphiregulin, HB-EGF (heparin-binding EGF-like growth factor), epiregulin, epigen, betacellulin, NRG-1 (neuregulin-1), NRG-2, NRG-3, NRG-4, NRG-5 and NRG-6. EGF family growth factors bind to the specific receptor family named as EGFR which includes four members: EGFR (ErbB1), ErbB2, ErbB3 and ErbB4 (Cohen, 1964, Harris *et al.*, 2003, Normanno *et al.*, 2005, Cohen, 2008, Higashiyama *et al.*, 2008). Human EGF is a 60-kDa protein with 53 amino acid residues and three intramolecular disulphide bonds (Carpenter and Cohen, 1990, Harris *et al.*, 2003). The epidermal growth factor receptor is a cytoplasmic transmembrane protein encoded by the *EGFR* gene belonging to the HER (ErbB) family of RTKs (receptor tyrosine kinases). The structure of the EGFR receptor consists of an extracellular ligand-binding domain, a single transmembrane hydrophobic helix and a cytoplasmic carboxy-terminal motif, which is restricted to tyrosine kinase function. The EGFR receptor undergoes a conformational change, once the ligand binds to the extracellular domain, which permits homodimerisation or heterodimerisation with another activated receptor of the HER family. Following dimerisation, autophosphorylation occurs in the intracellular tyrosine residues and

triggers various signal transduction pathways. The Ras/Raf/MAPK pathway, PI3K/Akt pathway and JAK/STAT pathways have been identified as three major signalling pathways that are activated by EGFR. Once activated, these pathways promote normal tissue homeostasis and over-activation may progress to a malignant cellular phenotype, including resistance to apoptosis, enhanced proliferation, invasion, metastasis and stimulation of angiogenesis in many cancer including HNSCC (Hackel *et al.*, 1999, Moghal and Sternberg, 1999, Nicholson *et al.*, 2001, Hynes and Lane, 2005, Bublil and Yarden, 2007, Uribe and Gonzalez, 2011). Overexpression of EGFR and TGF-α mRNA are prevalent and found to be elevated in 92% and 87% of HNSCC cases (Grandis and Tweardy, 1993). Studies also revealed that EGFR protein is overexpressed in 38-47% HNSCC (Bei *et al.*, 2004, Ongkeko *et al.*, 2005). Upregulation of EGFR have been seen in the normal epithelium adjacent to tumour dysplasia in late-stage tumours and in poorly differentiated tumours which supports the field cancerisation hypothesis in HNSCC (Grandis and Tweardy, 1993, Shin *et al.*, 1994). A study showed that EGFR expression was found to be low in laryngeal cancer as compared with tumours from the pharynx and oral cavity and suggested that EGFR expression may be site-specific (Takes *et al.*, 1998). Several studies suggested that other ErbB family members in addition to EGFR are overexpressed in HNSCC (ErbB2, 3% to 29%; ErbB3, 21%; ErbB4, 26%) (Bei *et al.*, 2001, Bei *et al.*, 2004, Schartinger *et al.*, 2004). EGFR, ErbB2, and ErbB4 were upregulated in invasive and *in situ* carcinoma, whereas ErbB3 was upregulated only in invasive carcinoma (Bei *et al.*, 2001). In most of these cases, two or more of these

receptors were upregulated concurrently, proposing the development of receptor heterodimers (Kalyankrishna and Grandis, 2006).

1.7.2 Transforming Growth Factor-beta (TGF-β) superfamily

The transforming growth factor-beta superfamily of proteins contains the TGF-β ligands (TGF-β1, 2, and 3), bone morphogenic proteins (BMPs) and activins/inhibins (Shi and Massague, 2003). TGF-β ligands bind to and induce signals through two types of transmembrane serine/threonine kinase receptors: TGFβR1 and TGFβRII. Seven TGFβRI and five TGFβRII family members have been defined so far (Massague, 2008). Canonical TGFβ signalling is mediated through SMAD family members which are grouped into three classes (receptor activated R-SMADs, inhibitory SMADs and the common SMAD4) and act as a transcription factors (Massague, 2008). After TGF-β binding, TGFβRI is phosphorylated by TGFβRII, which then phosphorylates and activates R-SMADs. TGFβ1, 2 and 3 signals through R-SMAD 2 and 3, whereas BMPs utilise R-SMAD 1, 5 and 8 (Massague, 2000). Activated R-SMADs then heterodimerise with SMAD4, translocate to the nucleus and control gene expression at SMAD binding elements (Massague *et al.*, 2005). Inhibitory SMADs mitigate TGFβ signalling by challenging R-SMADs for binding at TGFβRI and by engaging ubiquitin ligases to disintegrate TGFβRI and R-SMADs (Ebisawa *et al.*, 2001, Massague *et al.*, 2005). Ligand bound TGFβ receptors induce other signalling pathways such as the MAP kinases, PI3K/Akt and Rho-GTPases in

SMAD-independent non-canonical TGFβ signalling, which probably increase tumour growth after disruption of SMAD-dependent signalling (Zhang, 2009). TGFβ1 is overexpressed in around 80% of human HNSCCs and associates with more aggressive disease and worse survival (Lu *et al.*, 2004, Levy and Hill, 2006). Loss of functional TGFβ signalling triggers a compensatory amplification in TGFβ 1 production that ironically stimulates tumour growth by altering the tumour stroma, enhancing invasion, inflammation and angiogenesis in HNSCC (Lu *et al.*, 2006, Bian *et al.*, 2009, Bornstein *et al.*, 2009). Several studies have showed that TGFβRII may be down-regulated in around 70% of HNSCC (Lu *et al.*, 2006) and is linked with poorly differentiated tumours and more aggressive clinical behaviour (Muro-Cacho *et al.*, 1999, Fukai *et al.*, 2003). Not much is known about the association between TGFβRI loss and clinical behaviour in HNSCC, yet TGFβRI mutation has been discovered in around 19% of HNSCC metastases (Chen *et al.*, 2001). X-J Wang group revealed in their study that 86% of tumours and 67% of adjacent non-malignant mucosa in HNSCC had more than 50% SMAD4 reduction, proposing that SMAD4 down-regulation is an early event in HNSCC development (Bornstein *et al.*, 2009).

1.7.3 The Fibroblast Growth Factor (FGF) family

The FGFs are heparin-binding proteins and associations with cell-surface-associated heparan sulphate proteoglycans (HSPGs) have been shown to be vital in FGF-mediated intracellular signalling (Ornitz and Itoh, 2001). To date, 22 structurally

related FGF family members have been identified in humans (Eswarakumar *et al.*, 2005). FGF binds to a group of FGF receptors (FGFR1- 4) to transduce signals in target cells which share a common domain structure. HSPGs are linked with FGFR to regulate the binding of FGF to FGFR (Itoh and Ornitz, 2011). The FGFRs are comprised of three Ig-type extracellular domains, a transmembrane domain and a cytoplasmic tyrosine kinase domain (Beenken and Mohammadi, 2009, Turner and Grose, 2010). *FGFR* genes are regarded as proto-oncogenes activated in various human cancers as a result of point mutation, gene amplification and chromosomal translocation. Various signal transduction pathways are activated through FGF/FGFR system such as Ras-MAPK, PI3K/Akt, DAG-PKC and IP3/Ca^{2+}. FGF signals are engaged in anti-apoptosis, proliferation, drug resistance, angiogenesis, EMT (epithelial to mesenchymal transition) and invasion (Wesche *et al.*, 2011, Katoh and Nakagama, 2014).

FGFR1 gene amplification and protein overexpression have been observed in 17% and 12% of oral squamous cell tumours, respectively (Freier *et al.*, 2007). Several studies also revealed that, FGFR1/2/3 are overexpressed in 12-100% of HNSCC tissues (Drugan *et al.*, 1998, Wakulich *et al.*, 2002, Hase *et al.*, 2006, Freier *et al.*, 2007, Marshall *et al.*, 2011). While elevated FGFR1 expression has only been found in 12% of HNSCC cell lines, FGFR2 and FGFR3 overexpression has been found in the majority of HNSCC cell lines (Henson and Gollin, 2010, Marshall *et al.*, 2011). A decrease of FGFR3 levels in HNSCC cell lines leads to a 35% reduction in proliferation *in vitro* (Henson and Gollin, 2010).

1.7.4 The Vascular Endothelial Growth Factor (VEGF) family

Vascular endothelial growth factor (VEGF) represents a family of homodimeric glycoproteins, which at present comprises five mammalian and one parapox-virus encoded member. The VEGFs bind to three VEGF-receptor tyrosine kinases in an overlapping manner. Binding of VEGF to the receptors stimulates receptor dimerisation and activation and thus, induces signal transduction that causes cellular activities (Cross *et al.*, 2003). VEGFs were initially discovered as vascular permeability factor, VPF, a factor released by tumour cells that stimulates vascular leakage (Senger *et al.*, 1983). It is now evident that VPF corresponded to a biological function attributable to a family of polypeptide growth factors controlling blood and lymph vessel formation during embryonic development, providing vessel homeostasis and in wound healing in adult organisms. Imbalanced formation of blood and lymphatic vessels caused by excessive or decreased production of VEGF, results in many human diseases. VEGFs particularly interact with differentiating and mature endothelial cells and hematopoietic and endothelial precursor cells like angioblasts (Grunewald *et al.*, 2010).

VEGF-A (commonly termed as, VEGF) is a heparin-binding, dimeric polypeptide, which belongs to the platelet-derived growth factor family previously known as VPF. Besides VEGFA there are other members of this family which includes placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E (Senger *et al.*, 1983, Tischer *et al.*, 1989, Maglione *et al.*, 1991, Joukov *et al.*, 1996, Lee *et al.*, 1996, Olofsson *et al.*, 1996, Ferrara and Davis-Smyth, 1997, Achen *et al.*, 1998, Meyer *et al.*,

1999). Among these members, VEGF plays a vital role in vasculogenesis and angiogenesis (Beck and D'Amore, 1997). The human VEGF gene is organised in eight exons, separated by seven introns and its coding region spans approximately 14 kb(Houck *et al.*, 1991, Tischer *et al.*, 1991). At least six VEGF isoforms with different number of amino acids are generated through alternative exon splicing: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ (Robinson and Stringer, 2001) (Figure 1.26). $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ are the predominant form. The isoforms vary in their capacity to bind to the extracellular matrix (ECM) and heparin sulphate (Cross *et al.*, 2003).

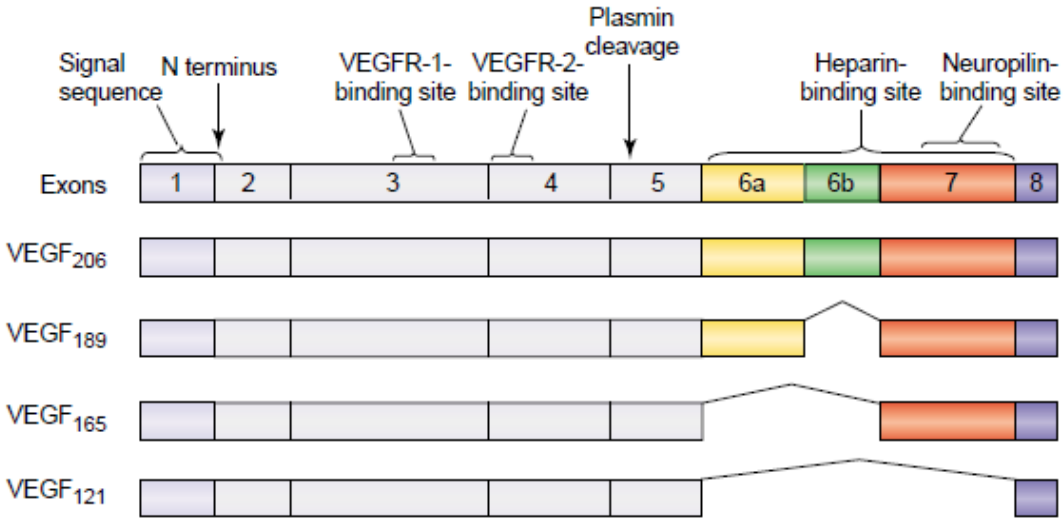


Figure 1.27 Various isoforms of VEGF.
Alternative exon splicing causes at least six human VEGF isoforms of which $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ are the predominant form (Cross *et al.*, 2003).

$VEGF_{121}$ lacks the region encoded by exons six and seven, so does not bind to heparin sulphate and is freely diffusible. It is a 28.4 kDa disulfide-linked

homodimeric protein consisting of two 121 amino acid polypeptide chains .$VEGF_{165}$ binds to cell surface heparin sulphate proteoglycans and $VEGF_{189}$ is almost completely sequestered in the ECM making a reservoir of VEGF that can be mobilised through proteolysis (Cross *et al.*, 2003, Ferrara *et al.*, 2003). The secreted forms of VEGF can stimulate the proliferation of endothelial cells and *in vivo* angiogenesis, on the other hand, the heparin-binding forms of VEGF can bind to the cell surface and ECM and enable the release of other angiogenic factors stored on heparin sulphate from the ECM (Christopoulos *et al.*, 2011).

Initially VGEF receptors were identified on the cell surface of vascular ECs *in vitro* and *in vivo*. Consequently, it became obvious that VEGF binding sites also occurs on bone-marrow derived cells and in cancer cells (Ferrara and Davis-Smyth, 1997, Shibuya, 2013). VEGF binds with two receptor tyrosine kinases (RTKs), VEGFR1 (Flt1) and VEGFR2 (Flk1/KDR). Both of these receptors have seven extracellular Ig-like domains, a single transmembrane region and a consensus intracellular tyrosine kinase domain. These receptors are expressed in most blood vessel endothelial cells (Shibuya *et al.*, 1990, Terman *et al.*, 1991). In contrast, VEGFC and VEGFD bind to VEGFR2 and also to VEGFR3 (Flt-4) which are largely restricted to lymphatic endothelial cells (Karkkainen *et al.*, 2002). PlGF and VEGFB binds only with VEGFR1 (Anisimov *et al.*, 2013). Most evidence suggests that, VEGFR2 is the major mediator of angiogenesis, microvascular permeability, endothelial cell mitogenesis and survival. In contrast, VEGFR1 plays an inhibitory role by sequestering VEGF and preventing its interaction with VEGFR2 especially during early embryonic

development. Yet, VEGFR1 has a recognised signalling role in mediating monocyte chemotaxis. Both VEGFR1 and VEGFR2 may mediate chemotactic and survival signal in hematopoietic stem cells (Ferrara *et al.*, 2003). The role of different tyrosine kinase receptors on different cell types is summarised in Figure 1.27.

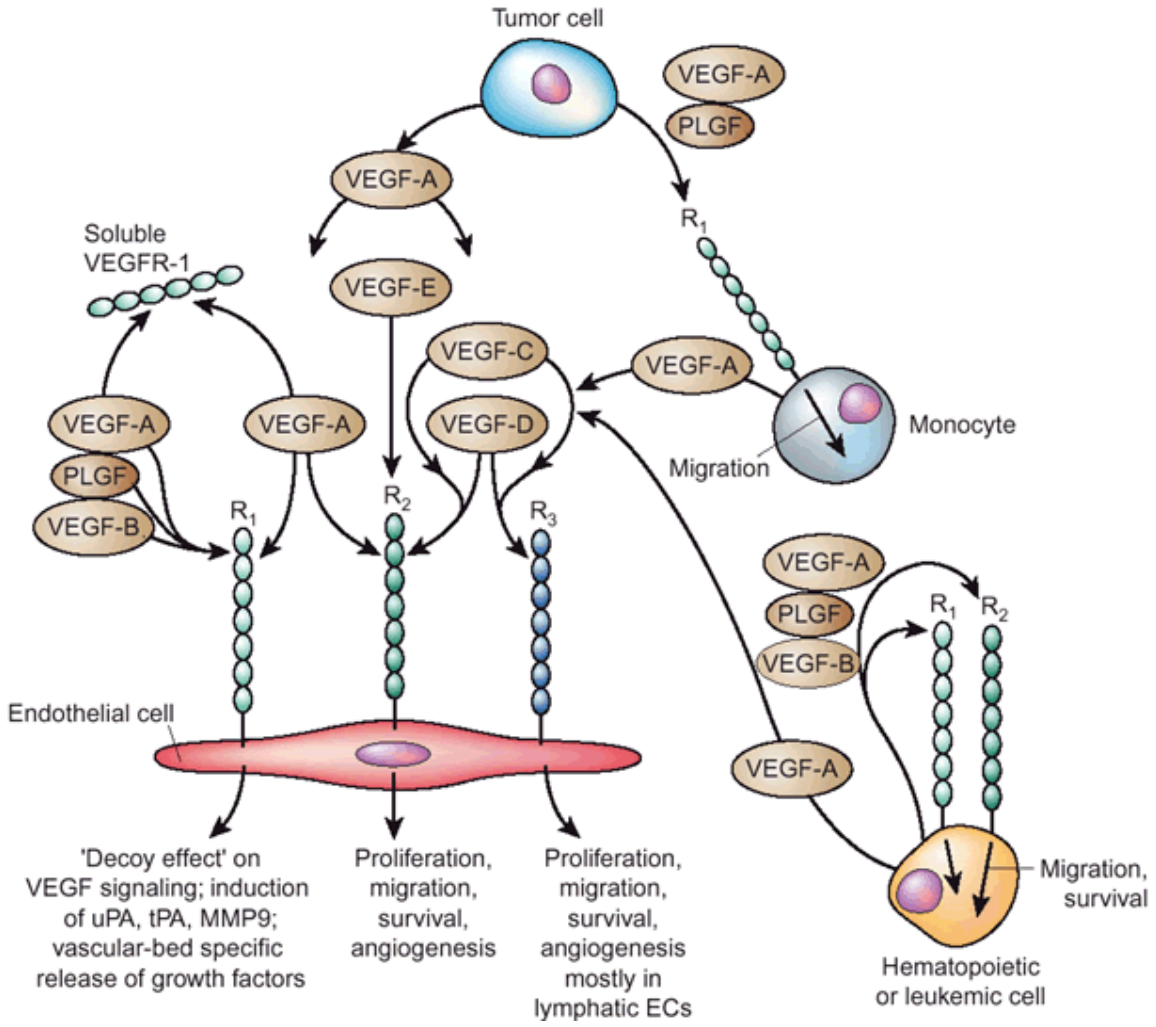


Figure 1.28 Different VEGF receptors function differently.

Varied activities of VEGF receptors largely depends on the ligands binds to them and on different cell types (Ferrara *et al.*, 2003).

It is now a well-accepted fact that angiogenesis is indispensable for the proliferation and metastasis of solid tumours, including head and neck squamous cell carcinoma. VEGF and its receptors are the main factor responsible for angiogenesis (Hoeben *et al.*, 2004). It has been confirmed that VEGFRs are also existent on tumour cells themselves and other components of tumour microenvironments, other than tumoural endothelial cells. Therefore, abundant and different interactions take place between these cells mediated via autocrine/paracrine pathways that stimulate angiogenesis, uncontrolled growth and metastasis (Mittal *et al.*, 2014). Consequently, assessment of VEGF expression and its receptors became a dependable prognostic tool in HNSCCs predicting the poor survival and metastasation (Margaritescu *et al.*, 2009). However, studies regarding VEGF-expression in HNSCC and the potential role of cancer progression are contradictory. The percentage of VEGF-positive OSCC cases differs from 25% to 100% with mean positivity of 78% (Margaritescu *et al.*, 2009). Table 7 summarise the data collected from the studies that shows the differential expression of VEGF isoforms and their associated receptors in HNSCC.

Table 7 Overexpression of different VEGF isoforms and their receptors in HNSCC

VEGF/VEGFR Type	**Cell type /region /stage in HNSCC**	**References**
A	Invasion depth	(Kyzas *et al.*, 2005a, Chuang *et al.*, 2006)
C, D	Infiltrating tumour front	(Shintani *et al.*, 2004)
A	Necrotic region	(Shweiki *et al.*, 1992, Mohamed *et al.*, 2004)

B, C > A	OSCC	(Shintani *et al.*, 2004)
C, R3	OSCC	(Fernandez Pujol *et al.*, 2001)
C	Perivascular stroma	(Zangani *et al.*, 1999, Ziegler *et al.*, 1999, Fernandez Pujol *et al.*, 2001)
C	Early stage	(Shintani *et al.*, 2004, Ohno *et al.*, 2007, Warburton *et al.*, 2007, Faustino *et al.*, 2008, Siriwardena *et al.*, 2008)
Decreasing C	Superficial to deep invasion	(Ohno *et al.*, 2007)
Increasing C	Superficial to deep invasion	(Nakaya *et al.*, 2005)
R1, R2, R3	OSCC	(Neuchrist *et al.*, 2001, Lalla *et al.*, 2003, Kyzas *et al.*, 2005b)
R3	Endothelial cells	(Matsuura *et al.*, 2009, Sugiura *et al.*, 2009)
R1, R2, R3	Macrophage	(Lalla *et al.*, 2003)
R1, R3	Fibroblast	(Lalla *et al.*, 2003)

Several studies have shown that overexpression of VEGF is associated with worse survival (Kim *et al.*, 2006, Tse *et al.*, 2007, Shao *et al.*, 2008, Joo *et al.*, 2009), whereas two studies found no correlation with these two factors (Salven *et al.*, 1997, Li *et al.*, 2005b). Most studies regarding the association between VEGF overexpression in HNSCC and clinicopathological predictors (e.g., TNM stage, tumour grade, tumour site, pattern of invasion, vascular invasion, lymph node metastasis, smoking, sex, age etc.) have also revealed contradictory results (Table 8).

Table 8 Association between VEGF overexpression in HNSCC and clinical parameter

Parameter	Correlated?	References
Differentiation	No	(Smith *et al.*, 2000, Kyzas *et al.*, 2005b, Shang and Li, 2005, Chuang *et al.*, 2006)
Differentiation	Yes	(Shintani *et al.*, 2004, Li *et al.*, 2005b, Johnstone and Logan, 2007)
Angiogenesis	No	(Tae *et al.*, 2000, Carlile *et al.*, 2001, Wong *et al.*, 2003)
Angiogenesis	Yes	(Arora *et al.*, 2005, Li *et al.*, 2005b, Shang *et al.*, 2006, Shao *et al.*, 2008, Joo *et al.*, 2009)
Clinical stage	Yes	(Uehara *et al.*, 2004, Kyzas *et al.*, 2005c, Shao *et al.*, 2008)
Clinical stage	No	(Maeda *et al.*, 1998, Shintani *et al.*, 2004, Chuang *et al.*, 2006)
Lymph node metastasis	Yes	(Tae *et al.*, 2000, Gallo *et al.*, 2002, Shang and Li, 2005, Chien *et al.*, 2006, Kim *et al.*, 2006, Joo *et al.*, 2009)
Lymph node metastasis	No	(Maeda *et al.*, 1998, Homer *et al.*, 2003, Uehara *et al.*, 2004, Kyzas *et al.*, 2005c, Li *et al.*, 2005b, Tse *et al.*, 2007)
Lymph node metastasis, recurrence, distant metastasis	Yes	(Shao *et al.*, 2008)
Lymph node metastasis, tumour grade, smoking	No	(Kyzas *et al.*, 2005c)

Note. Data from literature regarding the association between the clinical parameters and VEGF overexpression are disputed.

1.8 Signalling Pathways in HNSCC

There has been a recent upsurge in our understanding on how the flow of information through intercellular signalling networks controls cellular bioactivity. Indeed, the emerging knowledge of the basic mechanisms regulating intercellular and cell-to-cell communication is offering an extraordinary opportunity to understand physiological and pathological processes at the organismal, cellular and molecular levels, thereby recognising novel targets for pharmacological intervention in a multitude of diseases. In this regard, the evolving information on the nature of the deregulated signalling mechanisms in HNSCC and their potential involvement to disease progression is discussed below.

1.8.1 p53/Rb pathway

The tumour suppressor protein p53 maintains genomic stability by regulating cell cycle and growth arrest, DNA repair or apoptosis (Vogelstein *et al.*, 2000). p53 can arrest the cell cycle and activate repair or initiate apoptosis in response to DNA damage. Mouse double minute 2 homolog (MDM2), an E3 ubiquitin protein ligase can bind to p53 and causes its degradation. $p14^{ARF}$ is encoded by the gene CDKN2A, inhibits MDM2, thus protecting p53 from degradation (Vogelstein *et al.*, 2000, Sherr and McCormick, 2002). ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and rad3-related) pathways activate p53 by sensing DNA damage and phosphorylating the cell cycle checkpoint kinases CHK1 and CHK2. p53 thus trans-

activates a number of proteins which are involved in apoptosis and cell cycle arrest (Brady *et al.*, 2011).

Retinobalstoma (Rb) is also regarded as the tumour suppressor protein that regulates the expression of genes which are involved in cell cycle progression through the G1 restriction point. pRb inhibits E2F transcription factors that induce expression of S-phase genes and cell proliferation. Cyclin D1/CDK4/CDK6 complexes phosphorylates and activates pRb, resulting in the release of E2F. $p16^{INK4A}$ and p21 binds to the complexes, inhibits them and prevents them from phosphorylating pRb (Di Fiore *et al.*, 2013).

Limitless replicative potential and immortalisation can result from the mutations in p53 and pRb pathways. It is reported that in around 50-80% cases of HNSCC, mutation in TP53 gene occurs which is one of the earliest and most frequently detectable genetic alteration. p53 mutation can also be detected in premalignant dysplastic lesions and in histopathologically negative tumour margins (Brennan *et al.*, 1995b, Poeta *et al.*, 2007, Ebrahimi *et al.*, 2008). Use of tobacco and alcohol and increased TP53 mutations are closely associated in HNSCC and this association increased the risk of progression to cancer (Boyle *et al.*, 1993, Brennan *et al.*, 1995a). Mutations (7-9% cases of HNSCC) and copy number losses (20-30% cases of HNSCC) of CDKN2A gene inactivates pRb pathway and is observed early in the carcinogenesis of HNSCC (Agrawal *et al.*, 2011, Stransky *et al.*, 2011, Gaykalova *et al.*, 2014). The CCND1 gene, which encodes cyclin D1 in overexpressed in over 80% of HNSCC (Smeets *et al.*, 2006). Reduced survival in HNSCC is linked with TP53

mutation, cyclin D1 overexpression and p16INK4A loss (Bova *et al.*, 1999, Poeta *et al.*, 2007).

1.8.2 Ras/Raf/MEK/MAPK pathway

Guanosine nucleotide binding protein, Ras is a proto-ocnogene. There are three Ras genes: HRAS, KRAS and NRAS, the proteins are localised on the plasma membrane. When Ras is bound to guanosine diphosphate (GDP) it regarded as inactive. Upon activation Ras converts to GTP (guanosine triphosphate) bound form. Thus Ras-GTP forms then binds to and activates Raf-1. Raf-1 then phosphorylates MEK1 and MEK2 kinases which in turn activate the MAPkinases ERK1 and EKR2. These kinases translocate to the nucleus and target genes which are involved in cell growth, proliferation and survival (Bos, 1989, Schmukler *et al.*, 2014). HRAS mutation has been found in 4-5% of HNSCC cases (Agrawal *et al.*, 2011, Stransky *et al.*, 2011).

1.8.3 NOTCH pathway

NOTCH1 signalling is responsible for a variety of biological activities including survival, differentiation and self-renewal capacity. This pathway consists of two families of ligands, Delta-like 1, 3, 4 and Jagged 1, 2 and four receptors, NOTCH 1-4. Upon ligand binding, TNF-α converting enzyme (TACE) and γ–secretase cleaves NOTCH1 in two parts and releases NOTCH1 intracellular domain (NCID). NCID then translocates to the nucleus and induces transcription of *HRT* and *NES* family target genes. NOTCH1 is partly degraded by the ubiquitination pathway that involves *FBXW7* (Egloff and Grandis, 2012). A recent whole-genome sequencing

study revealed that the *NOTCH1* gene mutation is the second most common mutation in HNSCC, accounting for 14-15%. Mutations in other NOTCH family members account for 3-5% of HNSCC (Agrawal *et al.*, 2011, Stransky *et al.*, 2011). A new finding regarding *FBXW7* reported that this gene is mutated in 5% of HNSCC cases (Agrawal *et al.*, 2011). A recent study has also suggested that chromosomal aberrations in NOTCH signalling modulators JAG1, JAG2, MUMB and MAML1 are frequent along with the mutations in NOTCH itself (Pickering *et al.*, 2013).

1.8.4 JAK/STAT pathway

The JAKs are non-receptor tyrosine kinases and they bind with the cytokine receptor of the plasma membrane. Upon binding, receptors are activated by transphosphorylation. Activated cytokine receptors then employ STAT protein which is phosphorylated by JAKs, facilitating dimerisation and translocation to the nucleus to activate transcription of their target genes. The RTKs (receptor tyrosine kinases) such as EGFR can also phosphorylate JAKs which then activates the Ras and PI3K pathways. The JAK/STAT pathway is responsible for the bioactivities such as cell proliferation, survival, angiogenesis and inhibiting immune surveillance (Aaronson and Horvath, 2002). A study suggests that STAT3 is overexpressed in both cultured HNSCC cells and tumour specimens (Grandis *et al.*, 2000). Increased STAT3 expression is associated with increased Ras in tobacco-associated HNSCC and STAT5 promotes tumour growth, EMT and resistance to treatment-induced apoptosis in HNSCC (Xi *et al.*, 2003, Arredondo *et al.*, 2006, Koppikar *et al.*, 2008).

1.8.5 NFkB pathway

NFkB is a transcription factor which has five family members: p50 (NFkB1), p52 (NFkB2), p65 (RELA), c-Rel (REL) and RelB (RELB). NFkB is assembled by the polymerisation of these five members and upon activation translocates to the nucleus (Karin, 2006). Activation of NFkB involves the phosphorylation of IkB on Ser 32 and Ser36 residues. Phosphorylated IkB triggers rapid ubiquitination and degradation in the proteasome with consequent nuclear translocation and activation of NFkB. Activated NFkB participates in the expression of genes involved in inflammatory and immune responses, as well as in cell proliferation and survival (Hayden and Ghosh, 2008).The IkB kinase has a regulatory subunit, NEMO (IKKc) and two catalytic kinase subunits, IKKa (IKK1) and IKKb(IKK2) (Karin and Greten, 2005), all of which are activated by pro-inflammatory cytokines, tumour necrosis factor (TNF)-α and can readily be detected in HNSCC (Squarize *et al.*, 2006). Deregulation of NFkB stimulates tumour angiogenesis and metastasis and represses the apoptotic potential of chemotherapeutic agents and radiation, thus indicating treatment resistance (Karin and Greten, 2005, Nakanishi and Toi, 2005). The constitutive activation of NFkB is a common event in a variety of human cancers including HNSCC (Ondrey *et al.*, 1999, Karin and Greten, 2005). NFkB signalling plays a vital role at the early stages of HNSCC carcinogenesis because the expression and activity of NFkB is often upregulated and its protein levels increases gradually from premalignant lesions to invasive head and neck cancer (Ondrey *et al.*, 1999, Bindhu *et al.*, 2006, Mishra *et al.*, 2006, Sawhney *et al.*, 2007). A study also showed

that, NFkB stimulates the expression of the antiapoptotic protein Bcl-2 in HNSCC and restricting NFkB function leads to a significant reduction in cell survival and tumour growth in HNSCC (Jordan *et al.*, 1996, Chen *et al.*, 1999).

1.8.6 PI3K/Akt/mTOR pathway

The PI3K (phosphoinositide-3-kinases) pathway has appeared to be one of the most common targeted pathways in all sporadic human cancer, as proposed by the fact that mutation in one or more PI3K pathway components accounts for up to 30% of all cancers (Cully *et al.*, 2006). Genomic abnormalities such as mutation, amplification and rearrangements in a number of constituents of the PI3K signalling pathway, cause the deregulation of cellular growth and survival, which promotes a competitive growth advantage, metastatic potential and resistance to therapy (Hennessy *et al.*, 2005). According to the substrate preference and sequence homology PI3Ks are classified into three subclasses (I-III) (Cantley, 2002). The Class 1A PI3Ks are activated by EGFR or VEGFR and class IB PI3Ks are activated by GPCRs. Class IA PI3Ks consists of a p85 regulatory subunit and a p110 catalytic subunit. The P85 regulatory subunit binds and incorporates signals from a number of cellular proteins, including oncogenic proteins such as Ras and Src and growth factor tyrosine kinase-linked receptors, offering an integration point for activation of PI3K and its downstream signalling molecules (Cui *et al.*, 2014).

Activated PI3K converts the phospholipid component PIP2 (phosphatidylinositol 4, 5-bisphosphate) to PIP3 (phosphatidylinositol 3, 4, 5-triphosphate) by donating a

phosphate group. PIP3 is a component of the inner leaflet of the plasma membrane and serves as docking sites for proteins that contains PH (pleckstrin homology) domain, including Akt (protein kinase B) and PDK1 (phosphoinositide-dependent kinase 1). The Akt family of kinases has three family members; Akt1, Akt2 and Akt3. All three Akt isoforms retain two highly conserved threonine/serine phosphorylation sites that along with the PH domain are critical for Akt bioactivity. PDK1 phosphorylates Akt1 at Threonine 308 residue which lies in the T-loop of the catalytic domain, resulting in its activation (Alessi and Cohen, 1998, Cantley, 2002). The second phosphorylation residue (Serine 473) lies within a hydrophobic motif proximal to the C-terminus and is activated by a distinct protein kinase, most likely the mammalian target of rapamycin-rictor complex (mTOR complex 2) (Hennessy *et al.*, 2005, Sarbassov *et al.*, 2005). Strong evidence supports a vital role for Akt in the transmission of the pro-proliferative and transforming pathways induced by growth factors and oncogenes that stimulate PI3K (Luo *et al.*, 2003). Thus, the detection of Akt substrates has been the focus of many studies to determine the mechanisms by which this kinase controls insulin signalling, cell growth, apoptosis and cancer (Luo *et al.*, 2003). Amongst them, Akt inactivates pro-apoptotic factors including FOXOs (Forkhead transcription factors), BAD and procaspase-9, activates some transcription factors that upregulate anti-apoptotic genes, comprising NFkB, thus prevents cell death. It also inactivates p53 through MDM2 and activates CDK inhibitors p21 $^{CIP1/WAF1}$ and p27^{KIP1}, causing their elimination from the nucleus and subsequent cytoplasmic degradation, resulting in an increase in cell proliferation (Hennessy *et*

al., 2005). Akt also enhances β-catenin and cyclin D1 stabilisation by phosphorylating and inhibiting GSK-3β (glycogen synthase kinase-3 beta) (Vivanco and Sawyers, 2002). Akt is constitutively activated by frequent mutations in the small GTPase Ras, PI3K and receptor and non-receptor tyrosine kinases and inactivated by many tumour suppressor proteins like PTEN, TSC1, TSC2 and LKB1, highlighting the significant role of the dysregulation of the Akt pathway in cancer (Brazil *et al.*, 2004, Inoki *et al.*, 2005a).

Numerous works suggest that Akt is persistently activated in a number of HNSCC cases. Active phosphorylated Akt is identified in a vast majority of human HNSCC tissues and HNSCC-derived cell lines (Patel *et al.*, 2002, Amornphimoltham *et al.*, 2004). Furthermore, AKT activation is an early event in HNSCC progression, as it is identified in nearly 50% of tongue preneoplastic lesions (Massarelli *et al.*, 2005), and its activation signifies an independent prognostic marker of poor clinical outcome in tongue and oropharyngeal HNSCC (Massarelli *et al.*, 2005). Activating mutations and amplification in the PI3KI gene (PI3KCA) can be observed in around 10% and 40% cases of HNSCC, respectively (Woenckhaus *et al.*, 2002, Pedrero *et al.*, 2005, Murugan *et al.*, 2008). Additionally, lipid phosphatase, PTEN which inactivates PIP3 rapidly, is mutated or epigenetically inactivated in a large number of cancer including HNSCC, rivalling only p53 as one of the most significant tumour suppressor proteins (Sulis and Parsons, 2003). Loss of PTEN expression can be observed in around 30% of HNSCC cases and studies showed that this lack of expression may be an independent prognostic factor for poor clinical outcome (Lee

et al., 2001a, Squarize *et al.*, 2002). Thus, augmented Akt activity by the presence of several convergent pathways may explain why enhanced Akt function represents one of the most regular events in HNSCC (Molinolo *et al.*, 2007).

Despite the fact that Akt plays an important role in the development of HNSCC the downstream pathway(s) through which Akt acts, in this tumour type, is still not fully understood. Recent studies suggested that, tumour suppressor protein, TSC2 (tuberous sclerosis complex 2) is inactivated by Akt phosphorylation which associates with TSC1. This complex of TSC1/2 acts like a GTPase activating protein (GAP) for the Rheb1 (a small GTPase) (Inoki *et al.*, 2003, Inoki *et al.*, 2005b). The inactive TSC2 leads to the accumulation of active (GTP-bound) form of Rheb1 which in turn induces the phosphorylation and activation of mTOR (mammalian target of rapamycin) (Manning and Cantley, 2003). mTOR is an atypical serine/threonine kinase that phosphorylates p70-S6 kinase (eukaryotic translation regulators) and 4EBP1 (eukaryotic translation initiation factor 4E binding protein 1) (Hay and Sonenberg, 2004). Phosphorylated 4EBP1 then stimulates eukaryotic initiation factor 4E (eIF 4E) and ultimately results in increased translation from a group of genes that are involved in cell growth (Hay and Sonenberg, 2004). In HNSCC, it has been observed that eIF 4E gene amplification and protein overexpression are significantly associated with cancer progression and there is six-fold rise in risk of developing local recurrences in eIF 4E-positive surgical margins (Sorrells Jr *et al.*, 1999, Nathan *et al.*, 2002, Nathan *et al.*, 2004). Recent works by the S. Gutkind group have revealed that activation of mTOR is an early and one of the most frequent events in HNSCC

(Amornphimoltham *et al.*, 2005, Molinolo *et al.*, 2007, Molinolo *et al.*, 2009). Figure1. 28 below illustrate the most dysregulated signalling pathways in HNSCC.

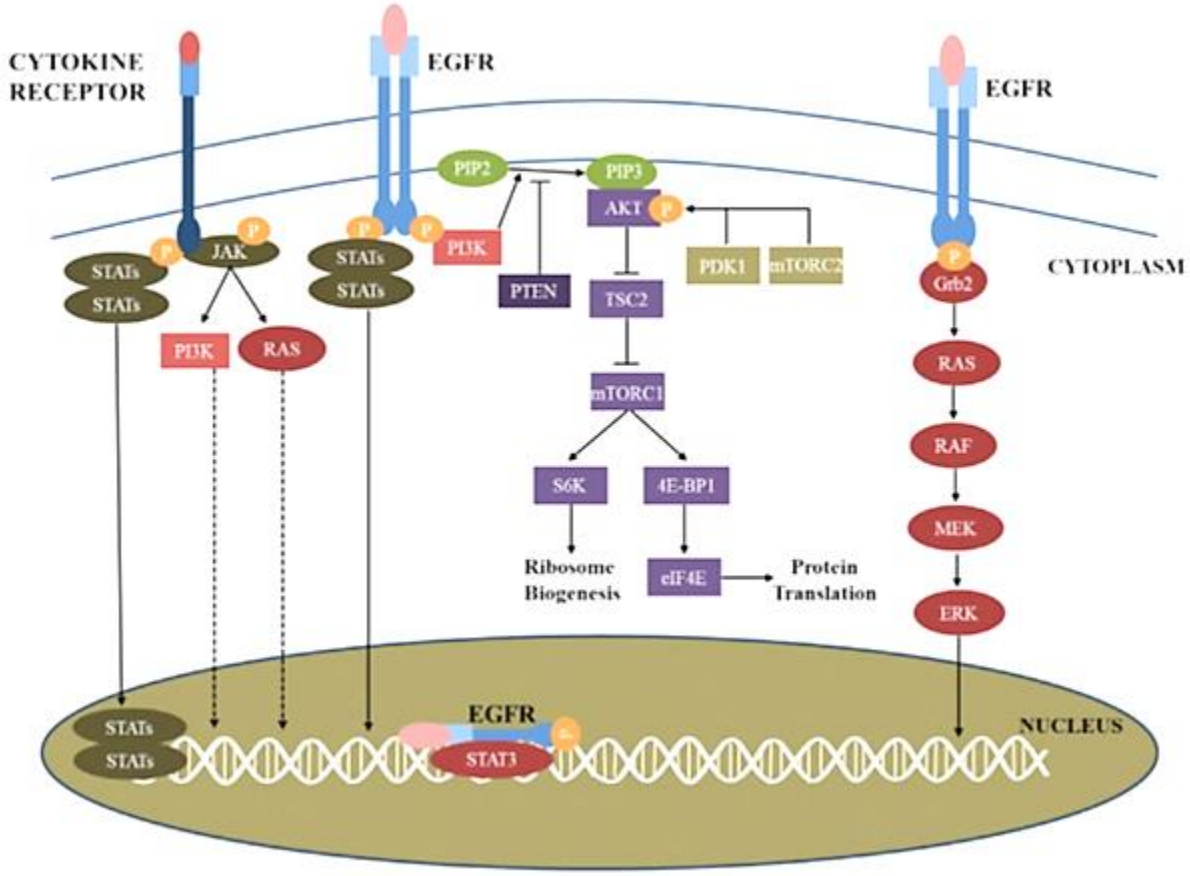


Figure 1.29 Dysregulated signalling pathways in HNSCC.

Ras/Raf, PI3k/Akt and JAK/STAT's are the most frequently altered signalling pathways in HNSCC (Bose *et al.*, 2013).

1.9 Akt and its role in metastasis

1.9.1 Metastasis cascade

Metastatic diseases remain the primary reason for cancer-related deaths (Siegel *et al.*, 2013). Whether it exists at the time of diagnosis, progresses during treatment, or happens at the time of disease relapse, the spreading of tumour cells from the primary lesion is the main reason for the mortality and morbidity of cancer patients (Palmer *et al.*, 2011). The metastatic process comprises a series of successive, interrelated steps including: detachment of tumour cells from the primary tumour and invasion of neighbouring, healthy tissue, intrusion into the blood and lymphatic vessels, circulation through the bloodstream (circulating tumour cells) to other sites and tissues in the body, extravasation from the vessel of distribution, and metastasis growth in specific distant organs and building a secondary tumour (Figure 1.29) (Woodhouse *et al.*, 1997, Leber and Efferth, 2009, Robert, 2013).

Many of these stages are dependent on cell motility and invasion, which permit the cells to change location within the tissues. To spread within the tissues, tumour cells apply similar migration mechanisms with those that happen in normal or non-neoplastic cells during physiological courses such as embryonic morphogenesis, inflammatory immune responses, wound healing, and angiogenesis (Friedl and Brocker, 2000).

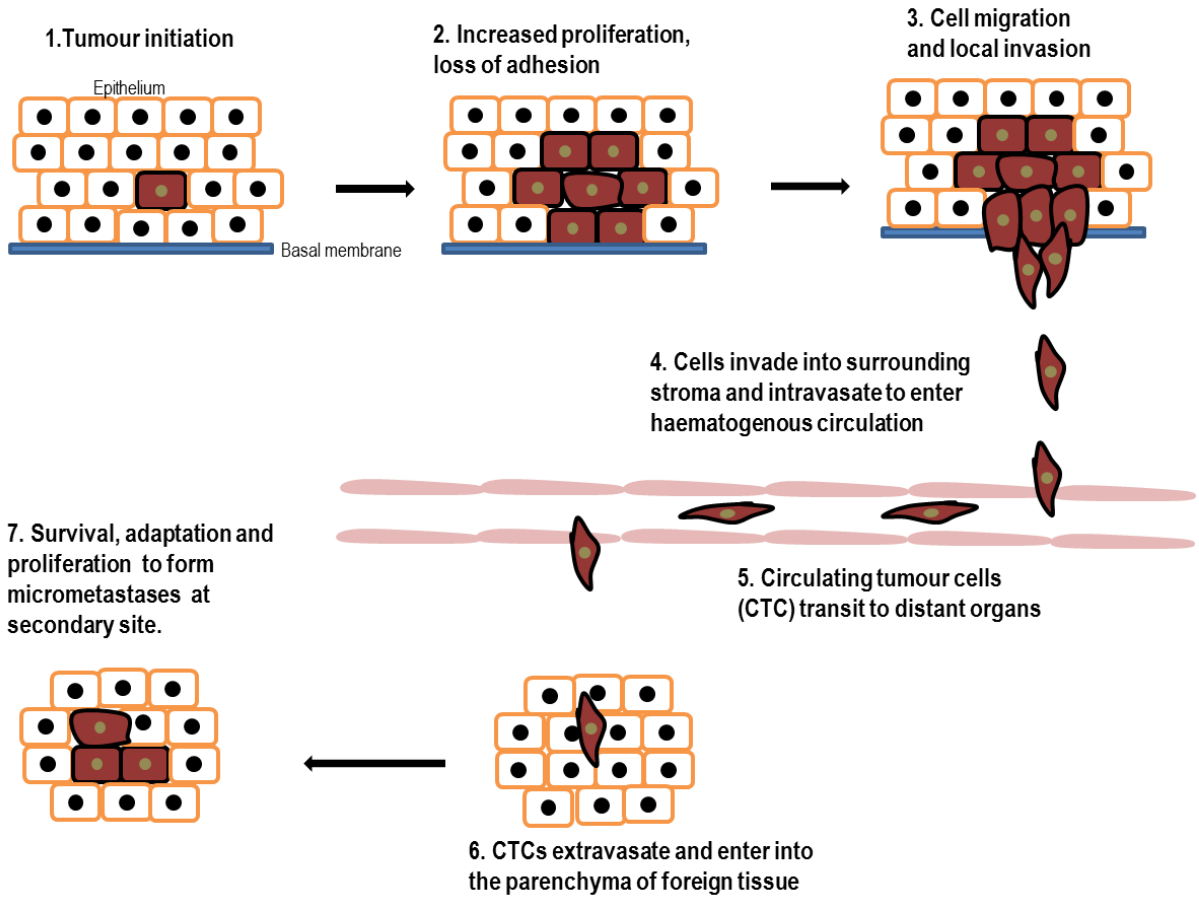


Figure 1.30 The metastasis cascade.

Sequential steps of metastasis include cell migration and invasion, intravasation, circulation, extravasion and homing.

Yet, distinct from the physiological processes of cell migration, tumour cell migration appears to be stimulated by a range of pro-migratory factors without responding stop signals, including autocrine motility factors generated by tumour cells, plus the soluble factors present at the secondary site (Friedl and Wolf, 2003, Zhou and Huang, 2011). Due to this imbalance of signals, cancer cells become unceasingly migratory and invasive, causing tumour expansion across tissue

boundaries and the development of cancer metastasis (Friedl and Wolf, 2003, Robert, 2013).

Cell migration through tissues results from highly integrated multistep cellular events (Lauffenburger and Horwitz, 1996, Friedl and Brocker, 2000, Ridley *et al.*, 2003). First, reacting to a migration-promoting agent, the moving cell polarises and elongates, extending protrusions in the way of migration. These protrusions, which can be spike-like filopodia, or large and broad lamellipodia, are typically driven by actin polymerisation and are steadied by adhering to the extracellular matrix or adjacent cells via transmembrane receptors associated with the actin cytoskeleton (Mitchison and Cramer, 1996). Consequently, forward extension of a lamellipodium and retraction of the trailing edge causes the translocation of the cell body (Mitchison and Cramer, 1996, Friedl and Wolf, 2003). Reorganisation of the actin cytoskeleton is the most important process of cell motility and is vital for most types of cell migration (Schmidt and Hall, 1998). In the course of cell migration, the actin cytoskeleton is dynamically remodelled, and this reorganisation creates the force indispensable for cell migration (Pollard and Borisy, 2003).

Variable experimental behaviour and histological patterns of tumour cells propose that tumour cells can utilise different cellular and molecular modes of migration based on cell-type specific autonomous mechanisms and reactive mechanisms stimulated by the local microenvironments (Ellis *et al.*, 2012). In malignant cancer patients, tumour cells are observed as both single cells and organised collective sheets indicating that cancer cells *in vivo* display the plasticity to switch between

single and collective cell migration. A range of studies on single cell migration develops a cellular and molecular basis which has furnished substantial understanding into the dissemination of the tumours whose cells migrate constitutively as single cells such as lymphomas and leukaemia or, after detachment from cohesive lesions through the epithelial to mesenchymal transition (EMT). Migration as collective sheets or clusters occurs when the junctions between cells are retained over an extended time period where cells are adherent to their neighbours. Although metastatic efficiency is enhanced by the transition to single cell migration, the basic requirements for the intra-vasation process can also be performed by a cell cluster as indicated by circulating grouped tumour cells from patient peripheral blood samples. Cell invasion is connected to and involves cell migration, but cells do more than migrate. Invasive cells degrade the extracellular matrix by ECM degradation and proteolysis and invade into neighbouring tissues (Ridley *et al.*, 2003, Friedl, 2004, Rorth, 2011, Inaki *et al.*, 2012).

Cell migration and invasion through tissues results from highly integrated multistep cellular events that are regulated by various signalling pathways, including the PI3K-Akt pathway. The serine/threonine kinase Akt (also known as protein kinase B or PKB) was initially discovered as a proto-oncogene. It plays a critical regulatory role in various cellular processes including, cancer progression, survival, insulin metabolism, protein synthesis etc. which is why it has become a focus of major attention. The Akt signalling pathway is activated by receptor tyrosine kinases (RTK), cytokine receptors, G-protein coupled receptor, integrin, B and T cell

receptors and other stimuli that stimulate the production of phosphatidylinositol 3,4,5 triphosphates (PtdIns(3,4,5)P3) through phosphoinositide 3-kinase (PI3K) (Figure 1.30).

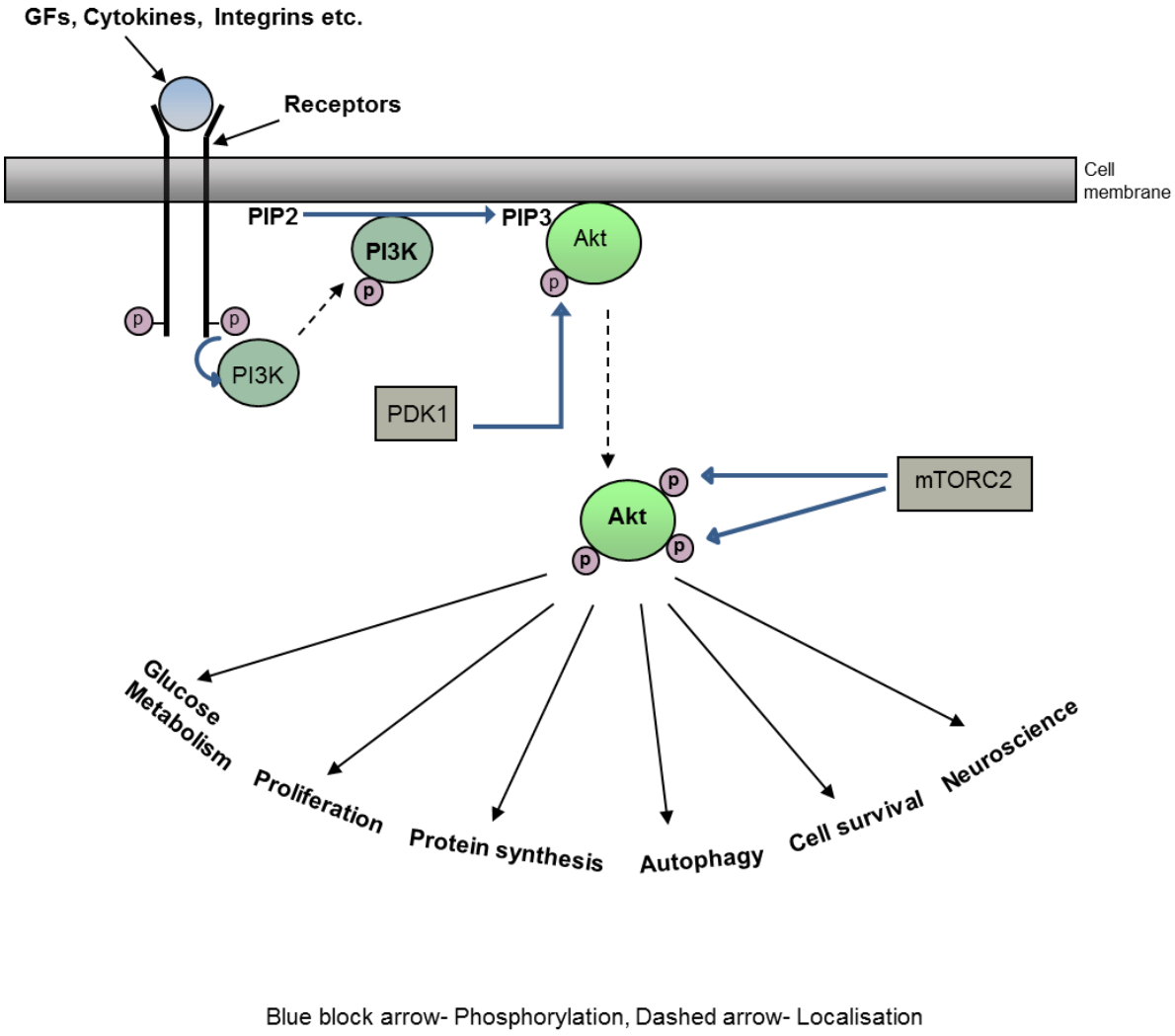


Figure 1.31 PI3K/Akt Signalling pathways.

The PI3k/Akt pathway is activated by many growth factors including VEGF. The central mediator of this pathway is Akt, which is activated or phosphorylated at Thr308 and Ser 473 residues by PI3K and mTOR C2, respectively. Once Akt is phosphorylated and activated, it is capable of phosphorylating multiple substrates generating diverse cellular processes, such as metabolism, proliferation, survival and protein synthesis.

The Class 1 PI3 kinases are a set of lipid kinases that phosphorylate the relatively abundant membrane phospholipid, phosphatidylinositol 4, 5 biphosphate (PIP2), generating small quantities of phosphatidylinositol 3, 4, 5 triphosphate (PIP3). This latter lipid signal controls a diverse set of effector molecules including the Akt group of oncogenic kinases termed as Akt1, Akt2 and Akt3. (Ellis *et al.*, 2010a). Activation of Akt1, a 60kDa kinase, depends on PI3K (Datta *et al.*, 1999). Increase of cellular PIP3 by PI3K eventually allows the activation of Akt1 by phosphorylation at T308 and S473 residues (Alessi and Cohen, 1998). This activation is completed by structural modification stimulated by PI3K-dependent kinase-1 (PDK-1)-dependent phosphorylation at T308 and stabilisation by mTORC2 or DNA-PK (DNA activated protein kinase) dependent phosphorylation at S473 (Feng *et al.*, 2004, Sarbassov *et al.*, 2005, Bozulic and Hemmings, 2009). A third phosphorylation site on Akt1 has been identified at T450 (Bellacosa *et al.*, 1998). This site is referred to as the turn phosphorylation site and is controlled by mTORC2 activity (Ikenoue *et al.*, 2008, Hart and Vogt, 2011). Activation of the three Akt isoforms plays a pivotal role in fundamental cellular functions by phosphorylating a variety of substrates. Recent studies have suggested that frequent alterations of the PI3K-Akt pathway occur in various types of human cancers. Constitutive activation of the PI3K-Akt pathway occurs due to amplification of the PIK3C gene encoding PI3K or the Akt gene, or as a result of mutations in components of the pathway, for example PTEN (phosphatase and tensin homologue deleted on chromosome 10), which inhibit the activation of Akt (Gonzalez-Angulo *et al.*, 2011, De Marco *et al.*, 2013, Wu *et al.*, 2013). Recent

evidence also suggested that Akt plays an important role in cancer cell migration and invasion (Yoeli-Lerner and Toker, 2006, Xue and Hemmings, 2013).

1.9.2 Akt in cytoskeletal rearrangements

The cytoskeleton is the cellular scaffolding or skeleton which is composed of a filamentous network of micro filaments (actin, myosin etc.), intermediate filaments (vimentin, keratin etc.) and microtubules (tubulin) (Frixione, 2000). The cytoskeleton is necessary for cellular structure maintenance, intracellular transport, cell division and many other functions. Cytoskeletal rearrangements occur in physiological events like cell movements and also in some pathological conditions like wound healing and cancer metastasis (Bonello *et al.*, 2012). Cellular motility either in physiological events or in pathological conditions is driven by cytoskeletal remodelling adapted by various signalling pathways. Migration potential arises because of a synergistic effect of all the three basic elements - filamentous actin, microtubule and the intermediate filament vimentin (Xue and Hemmings, 2013). Wide-ranging studies have focused on how stabilisation of intracellular filaments and dynamic polymerisation controls cell migration (Pollard and Borisy, 2003, Bugyi and Carlier, 2010). A broad spectrum of key factors associated with the cytoskeletal filaments are likely to be activated by Akt-mediated phosphorylation.

For the metastatic spread of cancer tissue, growth of the vascular network is important. The processes whereby new vessels form are called angiogenesis and this has an essential role in the supply of nutrients, oxygen and immune cells and also to

remove waste products (Folkman, 1995). Angiogenic factors are increasingly getting attention, especially in the field of neoplastic vascularisation. Vascular endothelial cell migration is a key step for angiogenic process. Microvascular endothelial cells display enhanced actin polymerisation upon stimulation by vascular endothelial growth factor (VEGF), linked with elevated motility and Akt activation. Inhibition of Akt activity by expression of a kinase-dead mutant abrogated actin bundle formation and blocked cell locomotion. This effect was increased when myristylated Akt was expressed (Morales-Ruiz *et al.*, 2000), demonstrating that Akt is a critical mediator of VEGF-induced endothelial cell migration through actin reorganisation. Data also suggests that eNOS activation via phosphorylation of Ser-1177 by Akt is necessary and sufficient for VEGF-mediated EC migration (Dimmeler *et al.*, 1999, Dimmeler *et al.*, 2000).

Interfering with Akt activity in chicken embryonic fibroblasts (CEF) also successfully blocked PI3K-transduced migratory signals. Overexpression of an active form of p70S6K1 was adequate to induce actin filament remodelling and cell migration in CEF cells, which requires Rac activity. This study establishes that activation of PI3K activity alone is sufficient to remodel actin filaments to enhance cell migration through the activation of Akt and p70S6K1 in CEF cells (Qian *et al.*, 2004). Another study showed, overexpression of the Integrin linked kinase (ILK) pathway is sufficient to induce PI3K dependent Rac1 activation. Inhibition of Akt, Rac1, or p70S6K1 inhibited the effects of ILK on actin filaments and cell migration, suggesting a regulatory role of the PI3K/Akt/p70S6K1/Rac1 signalling pathway in

response to ILK signalling (Qian *et al.*, 2005). In ovarian cancer, p70S6K1, downstream of PI3K/Akt pathway stimulated the rapid activation of Rac1 and cdc42 and their downstream effector molecule p21 activated kinase (PAK1). This regulates actin cytoskeleton in the metastatic phenotype (Ip *et al.*, 2011) . In neutrophils, activation of G-protein coupled receptors regulates F-actin polymerisation and cytoskeleton contraction through PIP3 signalling. This reorganisation pattern of actin ensures pseudopod extension in human neutrophils during chemo-attractant stimulation, which is dependent on Akt activity (Chodniewicz and Zhelev, 2003). Breast cancer cell migration and invasion often occurs in an Akt dependent manner which is characterised by enhanced filopodia production. A specific Akt inhibitor named API-2 (Akt phosphorylation inhibitor 2) blocks human breast cancer cell migration by blocking filopodia formation (Yang *et al.*, 2004). These observations of Akt activation and its role, suggest that Akt can potentially regulate cell migration through direct modulation of actin. Other studies showed that actin preferentially binds to phosphorylated Akt at pseudopodia with enriched bundles (Cenni *et al.*, 2003, Amiri *et al.*, 2007). Ho *et al* (2011) further confirmed that Akt can phosphorylate actin and therefore cortical reorganisation of actin associated with cell migration is strongly dependent on Akt activation (Ho *et al.*, 2011). Studies with HeLa cells revealed that Akt phosphorylates PAK1, a protein which belongs to the p21-activated serine/threonine kinase family and facilitates its binding with non-catalytic region of tyrosine kinase adaptor protein (Nck) and promote chemotaxis (Zhou *et al.*,

2003). This effect of Akt through PAK1 may be mediated by enhanced myosin 2 assembly and polarity (Chung *et al.*, 2001).

The actin-rich structure of highly motile cells such as invadopodia, filopodia and pseudopodia need to be stabilised to function properly. Actin-associated proteins are responsible for stabilising this actin structure by blocking the degradation of newly formed actin filaments (Chhabra and Higgs, 2007). One of the best example of this type of protein is APE (the Akt phosphorylation enhancer), also termed as girders of actin filaments (girdin). APE/girdin maintains the integrity of actin filaments, especially the actin meshwork at the leading edge of migrating cells. Decrease of APE/girdin destabilises the actin bundles, causing ablation of stress fibres and cortical actin structure. This leads to loss of directional migratory capacity and demonstrates the crucial activity of APE/girdin in the organised regulation of cell migration. Enomoto and colleagues established that APE/girdin is phosphorylated by Akt on serine 1416 (S1416) (Enomoto *et al.*, 2005). Upon stimulation by EGF, S1416 phosphorylation initiates the translocation of APE/girdin from the junctions between actin filament to the leading edge, co-localised with pAkt. This form of activation of APE/girdin is critical for cell migration. Indeed, recent observations have demonstrated that APE/girdin regulates actin reconstruction and Akt controlled cell motility in fibroblasts, human breast cancer and oesophageal squamous cell carcinoma (Jiang *et al.*, 2008, Weng *et al.*, 2010, Natsume *et al.*, 2012, Shibata *et al.*, 2013) . Akt has also been shown to promote actin organisation and cell motility

mediated by the mechano-protein and Akt-substrate ANKRD2 (Ankyrin repeat domain protein 2, also known as ARPP).

Actin-associated structural (cross-linker) protein, filamin A (Stossel *et al.*, 2001, Feng and Walsh, 2004) is phosphorylated by Akt on S2152 site (Ravid *et al.*, 2008) . In turn, phosphorylated filamin A mediates caveolin-1 induced cancer cell migration through the IGF signalling pathway (Ravid *et al.*, 2005, Nallapalli *et al.*, 2012).

Besides, insulin and PDGF induce the sodium-hydrogen exchanger isoform 1 (NHE1) which is a key mediator of stress fibre disassembly. Akt is shown to phosphorylate NHE1 on S648 and it has been suggested that it is critical for the growth factor induced cytoskeletal rearrangements that favours cell migration and invasion (Meima *et al.*, 2009). Different studies have demonstrated the migration of different cell types by modulating the cytoskeleton through NHE1 although the role of Akt was not elucidated (Denker and Barber, 2002, Stock and Schwab, 2006, Stuwe *et al.*, 2007, Martin *et al.*, 2011). Another study showed that NHE1 interacts with an actin regulatory protein, cortactin, in invasive breast cancer cells and stabilise the newly formed invadopodia (Magalhaes *et al.*, 2011). A recent study in fibroblast cells demonstrated that the Akt pathway is necessary for the translocation of NHE1 to the leading edge and actin nucleation at the lamellipodium that supports directional cell migration (Clement *et al.*, 2013).

Extensive studies were carried out to investigate the role of intermediate filaments in cell motility (Chang and Goldman, 2004, Helfand *et al.*, 2004). Vimentin, a type 3 filamentous protein is the most abundant intermediate protein that supports normal

cell and tissue integrity. Vimentin is shown to be highly expressed in motile cells both in physiological and pathological conditions. It is phosphorylated by Akt1 on S39, stabilised and thereby regulates cancer cell invasion in aggressive sarcoma (Zhu *et al.*, 2011). It has also been shown that vimentin is highly expressed in breast cancer and lung metastases (Lahat *et al.*, 2010, Satelli and Li, 2011) but the specific mechanism by which some of the Akt substrates control cell migration is still uncertain. For example, a component of E3 ligase named S-phase kinase-associated protein 2 (Skp2) is phosphorylated by Akt on S72, activates Skp-2 dependent ligase activity and stimulates cell migration through maintaining Skp2 in the cytoplasm (Gao *et al.*, 2009, Lin *et al.*, 2009). Akt also promotes cell migration by regulating microtubule dynamics through Akt/GSK3β axis-dependent activation of microtubule binding protein, APC (adenomatous polyposis coli) (Onishi *et al.*, 2007, McPhee *et al.*, 2008, Hong *et al.*, 2009).

Akt also interacts with pro-migratory proteins, in addition to targeting cytoskeletal proteins thus facilitating crosstalk between associated signalling axes. VEGFR/eNOS signalling pathway controlled cell migration is dependent on Akt mediated phosphorylation on S1177 (Dimmeler *et al.*, 2000). Accumulating evidence has pointed out the importance of NO in pathological situations, especially in malignant tumours (Xu *et al.*, 2002, Ridnour *et al.*, 2012). Furthermore, VEGFR signalling often cooperates with a G-protein coupled receptor named sphingosine-1 phosphate receptor 1(SIPR1, also known as endothelial differentiation gene 1, EDG-1). eNOS activates upon binding of SIP in SIP receptor 1. Thus SIP/SIPR1 activation leads to

the phospho-activation of VEGFR which is facilitated through phospho Src kinase, thus consequently activates the PI3k/Akt/eNOS axis (Spiegel and Milstien, 2003). Akt mediated phosphorylation of SIPR1 on T236 further enhanced this activity, stimulates cortical actin assembly, angiogenesis and chemotaxis (Lee *et al.*, 2001b, Ozaki *et al.*, 2003). Thus, Akt plays a central role in regulating VEGFR and SIP/SIPR1pathway and actively controls cell migration.

A member of the largest tyrosine kinase protein family named, EphA2 (Ephrin receptor tyrosine kinase A2) has also been shown to be phosphorylated by Akt on S897. In human brain cancer cells S897 phosphorylation is responsible for Akt mediated migration and invasion due to effects on dendritic actin cytoskeleton rearrangement and lamellipodia formation (Miao *et al.*, 2009, Pasquale, 2010). In a recent study, scientists have shown that EphA2 recruits Ephexin4 (a guanine nucleotide exchange factor for the small GTPase RhoG) upon phosphorylation on S896 and promotes breast cancer and colorectal cancer cell migration and anoikis resistance (Kawai *et al.*, 2013).

It is now well established that membrane redistribution of integrins by various signalling pathways is a critical mediator of cellular movement. ANK repeat and pleckstrin homology domain-containing protein 1 (ACAP1) is a GTPase activating protein (GAP) for ADP ribosylation factor 6 (ARF6) which participates in integrin β recycling. ACAP1 is phosphorylated by Akt on S554 and stimulates integrin recycling and therefore promotes cell migration (Li *et al.*, 2005a). Another GTPase activating protein, RhoGAP22 is shown to be phosphorylated by Akt on S16 upon

stimulation by insulin or possibly PDGF, playing a vital role in regulating cell migration, leading to modulation of Rac1 activity (Rowland *et al.*, 2011).

Several studies have demonstrated the role of mammalian target of rapamycin complex 1 (mTORC1) in cellular movement and their relationship with Akt (Berven *et al.*, 2004, Sakakibara *et al.*, 2005). Akt may positively regulate mTORC1 and in turn, increase the phosphorylation of p70S6K1 (S6K1) and inhibit eukaryotic initiation factor 4E binding protein 1(4EBP1). It has been published that a tumour suppressor gene, tuberous sclerosis complex 1/2 (*TSC1/2*) inhibits the S6K1 and activates 4EBP1 and this function of *TSC1/2* is mediated by inhibiting mTORC1. It has also been shown that TSC2 is inhibited by Akt-mediated phosphorylation which destabilises the complex and activates mTORC1 (Inoki *et al.*, 2002). In the single cell motility assay, IGF-1 stimulated cell motility was inhibited by down regulation of S6K1 by using lentiviral and by ectopic expression of constitutively hypophosphorylated 4EBP1 (Liu *et al.*, 2006a). S6K1 regulates cell motility, perhaps related to its regulating phosphorylation of the focal adhesion proteins, such as focal adhesion kinase (FAK), paxillin and $p130^{Cas}$, and F-actin reorganisation (or lamellipodia formation) (Liu *et al.*, 2008). Furthermore, mTORC1 mediates phosphorylation of ERK1/2 (extracellular-signal related kinase) on T202 through direct or indirect regulation of PP2A (Protein phosphatase 2A). Inhibition of PP2A activates EKR1/2 which promotes motility in a number of transformed cells and cancer cell lines (Benefield *et al.*, 1997, Jackson and Young, 2003, Wlodarski *et al.*, 2006, Liu *et al.*, 2010, Li *et al.*, 2013).

A number of studies have also demonstrated that transforming growth factor β1 (TGF β1) increases the migration of human chondrosarcoma and lung cancer cells through the PI3K/Akt signalling pathway. Akt activated IKKαβ (IkB kinase) can phosphorylate IkBα and p65 on S536 residue. NFkB thus dissociates from IkBα and activates β1 integrin and αvβ3 integrin which promotes human lung cancer and chondrosarcoma cell migration (Yeh *et al.*, 2008, Fong *et al.*, 2009).

Studies with human trophoblast cells have shown that abrogation of mTOR signalling, leading to the lack of functional mTORC1 is involved in a tightly regulated network of the intracellular JAK/STAT signalling pathway and contributes to the invasiveness of trophoblast cells by regulating matrix-remodelling enzymes such as MMP9 (matrix metalloproteinase), MMP2, uPA (urokinase plasminogen activator) and PAI-1 (plasminogen activator inhibitor) (Busch *et al.*, 2009, Zhou and Huang, 2011).

The opposing roles of Akt in cell migration have also been discussed in different studies. Akt phosphorylates kidney ankyrin repeat-containing protein (kank) which consequently lead to negative regulation of stress fibre assembly and RhoA activation, thus attenuating cell migration (Kakinuma *et al.*, 2008). An actin binding protein, palladin, phosphorylated by Akt1 on S507 inhibits breast cancer cell migration by disrupting F-actin bundles (Chin and Toker, 2010a). On the other hand, Akt2 contributes to palladin stability independent of S507 phosphorylation (Chin and Toker, 2010b). Similarly, Akt phosphorylates TSC2 (tuberous sclerosis complex

2), a Rho GTPase regulator that inhibits breast cancer cell migration due to impaired F-actin assembly (Liu *et al.*, 2006b).

1.9.3 Akt in EMT

Epithelial cells are tightly connected with their adjacent cells and with actin filaments via E-cadherin-containing adherent junctions and α or β-catenin, respectively. Before migrating as single cells, carcinoma cells must break these intercellular junctions and invade stromal tissues. To facilitate this type of cellular invasion epithelial tumour cells undergo a process termed as epithelial to mesenchymal transition (EMT). EMT can be stimulated either by extracellular cytokines, for example EGF, TGF α and β, FGF, or by intracellular cues, such as oncogenic Ras (Yang and Weinberg, 2008, Thiery *et al.*, 2009, Nieto, 2011). During EMT, epithelial cells gain mesenchymal phenotypes by losing their polarity and cell-cell contacts. Characteristics of EMT are the functional loss of E-cadherin and down-regulation of epithelial markers such as, cytokeratin and ZO-1, and the overexpression of mesenchymal or fibroblast markers N-cadherin, vimentin and fibronectin (Figure 1.31) (Larue and Bellacosa, 2005, Zheng and Kang, 2013).
EMT is reversible and sometimes, cells undergo the reciprocal mesenchymal to epithelial transition (MET). During the development process,

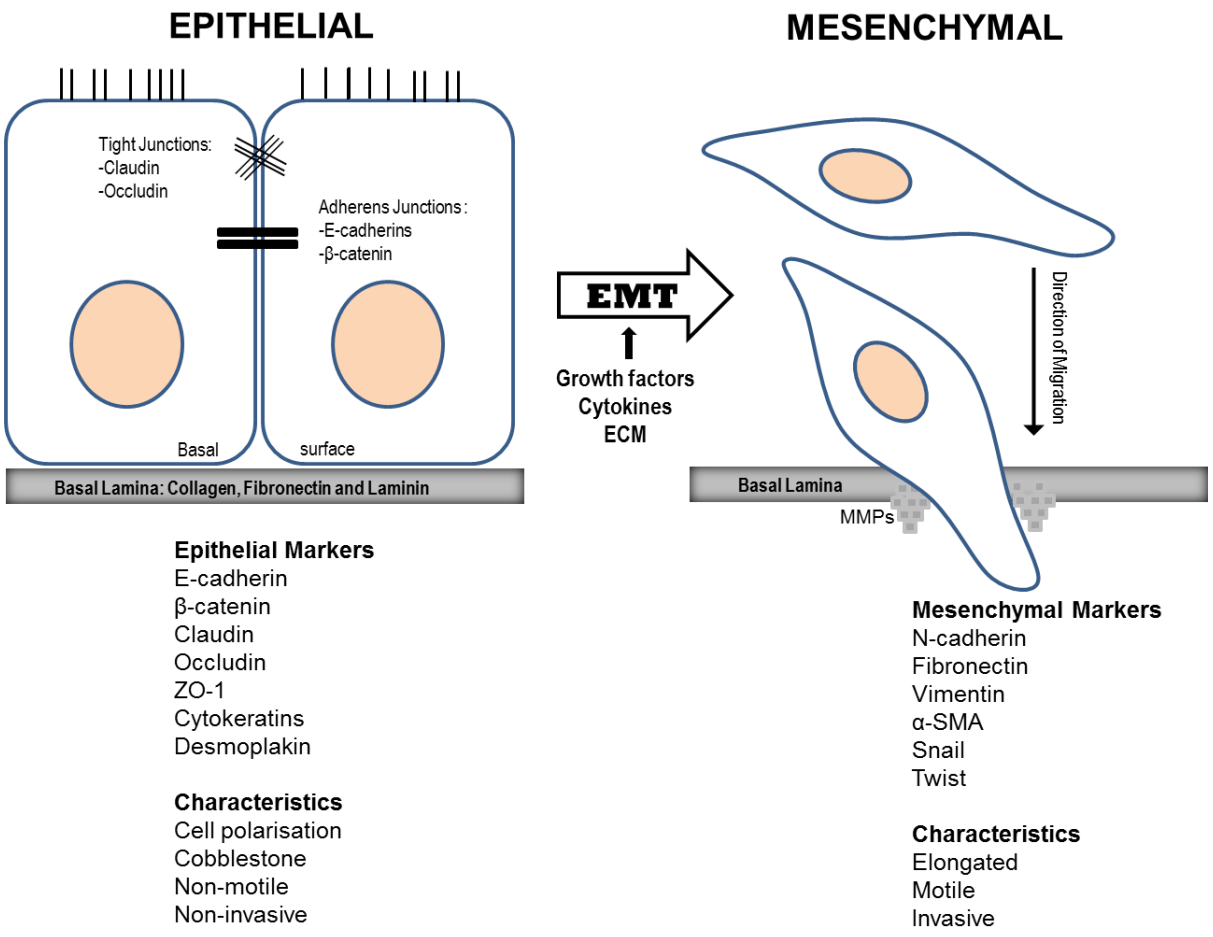


Figure 1.32 Epithelial to Mesenchymal Transition (EMT) and associated biological markers.

Upon growth factor stimulation epithelial cells undergo a morphological change to a mesenchymal phenotype, which is characterised by its motile and invasive behaviour.

EMT plays a vital role in the formation of various tissues and organs such as the neural crest, heart, musculoskeletal and peripheral nervous systems. Only a few cells in adult organisms hold the ability to undergo EMT in specific physiological or pathological events like wound healing. However, tumour cells often gain the ability to reactivate the EMT process during cancer progression and confer selective

advantages like enhanced migration and invasiveness (Thompson and Williams, 2008, Zheng and Kang, 2013).

A number of studies have reported that Akt is frequently activated in human carcinoma (Ringel *et al.*, 2001, Testa and Bellacosa, 2001, Fresno Vara *et al.*, 2004, Bellacosa *et al.*, 2005, Wu *et al.*, 2009). Akt2 has been shown to be activated in ovarian carcinoma affecting epithelial cell morphology, tumourigenicity, cell motility and invasiveness which is characterised by the loss of histological features of epithelial differentiation (Bellacosa *et al.*, 1995). The first study that reported the definitive role of Akt in EMT was published in 2003, in which squamous cell carcinoma lines overexpressing activated mutants of Akt were shown to undergo EMT and down regulate E-cadherin (Grille *et al.*, 2003). Loss of E-cadherin and relocalisation of β-catenin from the membrane to the nucleus is frequently detected in tumour cells undergoing EMT (Kalluri and Weinberg, 2009, Thiery *et al.*, 2009). A number of transcription factors have been identified that can commence and maintain this process, including Snail, Twist and Zeb. The definitive signalling mechanism of normal regulation of these factors at a molecular level are still poorly understood, yet they are apparently deregulated in many invasive cancers (Larue and Bellacosa, 2005, Bellacosa and Larue, 2010). Recent evidence suggests a strong relationship between Akt and EMT-inducing transcription factors. The first evidence was that Snail is phosphorylated by GSK3β (glycogen synthase kinase 3 beta) in normal epithelial cells but is very unstable and hardly detectable. Expression of Snail in epithelial cells strongly induces morphological change associated with enhanced

migratory capacity (Zhou *et al.*, 2004, Katoh and Katoh, 2006).

Hyperphosphorylation of Akt down regulates GSK3β activity depending on S9 phosphorylation. Because GSK3β-dependent phosphorylation of β-catenin and Snail leads to ubiquitination and degradation, down regulation of GSK3β results in the stabilisation and the nuclear accumulation of β-catenin and Snail. Nuclear Snail represses transcription of CDH1 gene encoding E-cadherin, to induce the EMT program. Stabilisation of Snail by phospho-inhibiting GSK3β also increases the expression of vimentin, N-cadherin and MMP-9. Nuclear β-catenin activates the transcription of cMYC and cyclin D1 gene, which also plays a significant role in the EMT. This is perhaps consistent with the situation in invasive cancers, in which high Akt phosphorylation leads to negative GSK3β expression and Snail overexpression. (Wu *et al.*, 2012, Ha *et al.*, 2013, Smith *et al.*, 2013, Wang *et al.*, 2013).

A transcription/translation regulatory protein named Y-box binding protein-1 (YB-1) is reported to be phosphorylated by Akt and translocated to the nucleus. Nuclear YB-1 thus activates Snail and reduces E-cadherin expression, which in turn promotes EMT in invasive breast carcinoma (Evdokimova *et al.*, 2009). Furthermore, upregulated Snail could in turn, increase Akt activity (Cho *et al.*, 2007). An unexpected role of Akt2 in transcriptional control is also revealed. Snail1 increases the binding of Akt2 to the E-cadherin (CDH1) promoter and Akt2 interference inhibits Snail1 repression of CDH1 gene (Villagrasa *et al.*, 2012). Akt2 could also be activated by another EMT-inducer, Twist, in invasive breast cancer cells (Cheng *et al.*, 2007). Inhibition of Akt also down-regulates Twist in cancer cells (Hong *et al.*,

2009). Furthermore, Akt phosphorylates and activates Twist1, which in turn enhances the phosphorylation of Akt because of increased TGFβ signalling in human breast cancer (Yao *et al.*, 2008, Xue *et al.*, 2012, Yokoyama *et al.*, 2012). Recent data also suggests that the polycomb group protein named B lymphoma Mo-MLV insertion region 1 homolog (Bmi1) is a downstream target of Twist1 and is crucial for EMT and cancer metastasis (Yang *et al.*, 2010). Akt, remarkably, could directly phosphorylate Bmi1 in high grade prostate tumours (Nacerddine *et al.*, 2012). Promotion of Akt activity by Bmi1 was also found to promote EMT by blocking GSK3β-mediated degradation of Snail in HNSCC and breast cancer (Song *et al.*, 2009, Guo *et al.*, 2011). Twist and Bmi1 also mediate suppression of the miR let-7i which results in NEDD9 and DOCK3 overexpression and promotes mesenchymal motility in HNSCC, melanoma and breast cancer via Rac1 (Ahn *et al.*, 2012, Sanz-Moreno, 2012, Yang *et al.*, 2012). In many cases breast cancer metastasis may be under the control of a balance between Akt1 and Akt2 and their link with MiR-200/Zeb/E-cadherin axis (Iliopoulos *et al.*, 2009, Hill *et al.*, 2013). Taken together, several studies demonstrate an important interaction between Akt and EMT inducer-associated signalling. This synergistic interaction has critical and unfortunate pathological effects: 1) it stops stress-induced cell cycle arrest in cancer cells, 2) it stimulates pro-invasive/metastatic gene expression, and 3) it maintains up regulation of PI3K/Akt signalling, which further increases the anti-apoptotic potential of cancer cells (Smith *et al.*, 2013, Xue and Hemmings, 2013, Zheng and Kang, 2013). Figure 1.32

summarises the role of Akt in metastasis with its possible association with substrates.

1.9.4 Akt in HNSCC metastasis

The term HNSCC (Head and Neck Squamous Cell Carcinoma) refers to epithelial tumours that arise in the oral cavity, pharynx, larynx and nasal cavity, with the main risk factors being alcohol and/or tobacco use and HPV infection (Gillison *et al.*, 2000, Neville and Day, 2002). It is the sixth most common type of cancer by incidence and around 500000 new cases a year worldwide (Kamangar *et al.*, 2006). It has recently been shown that Akt is persistently activated in the vast majority of HNSCC cases. Active forms of Akt (as phosphorylated) can readily be detected in both experimental and human HNSCCs and HNSCC-derived cell lines (Amornphimoltham *et al.*, 2004). Akt can be activated due to the overactivity of different growth factors, chemokines, integrin etc. and their respective receptors, ras mutations, PI3Ka gene amplification, overexpression, or activating mutations, together with aberrant PTEN activity due to genetic alterations or decreased expression in HNSCC (Amornphimoltham *et al.*, 2011). Akt activation is an early event in HNSCC progression which can be identified in as many as 50% of tongue preneoplastic lesions

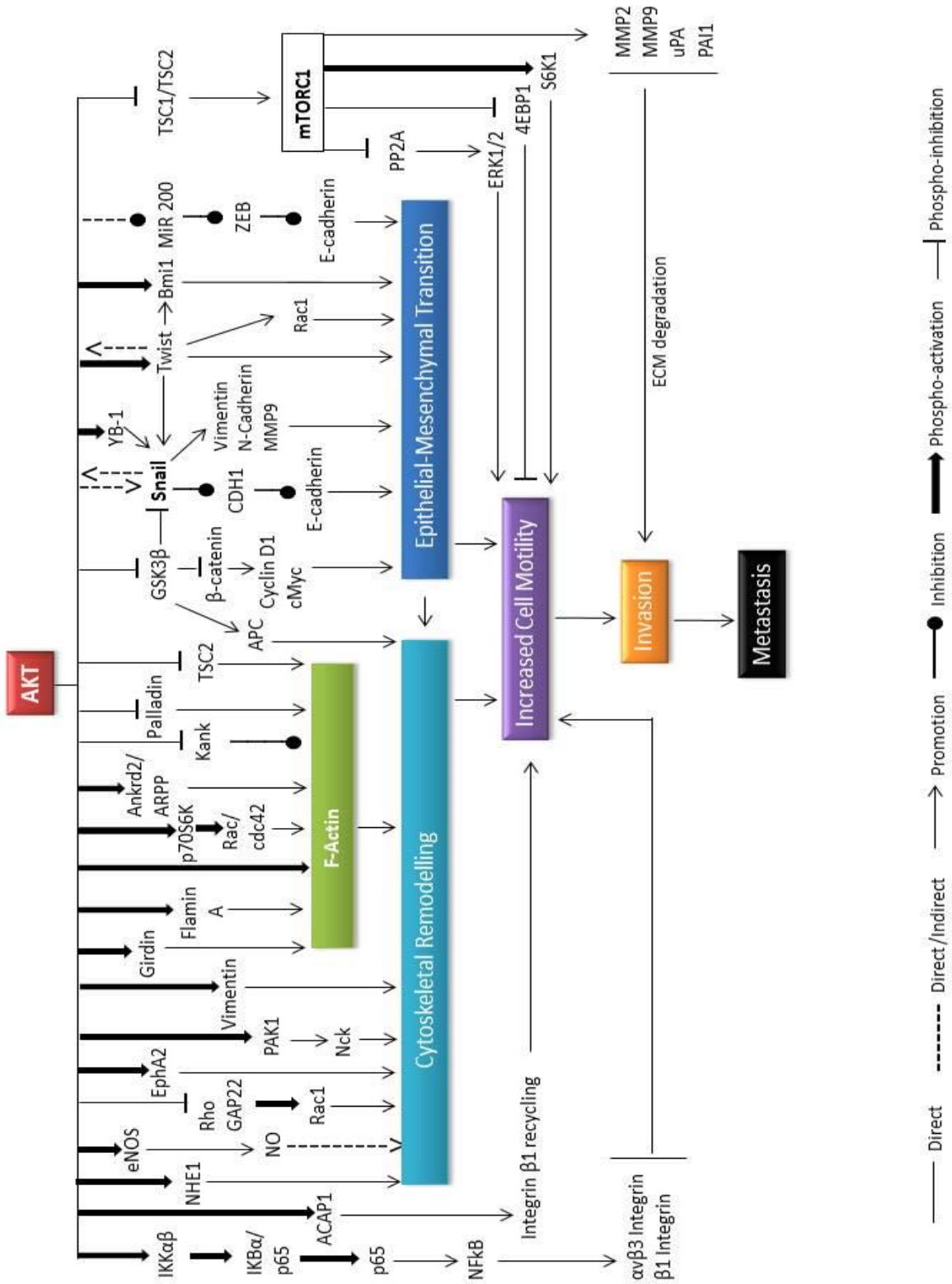


Figure 1.33 Role of Akt in metastasis

Phosphorylated Akt activates a range of signalling molecules responsible for cytoskeletal rearrangements and EMT. These two biological phenomena are the mechanism for cell migration and invasion.

Akt activation also represents an independent prognostic marker of poor clinical outcome in both tongue and oropharyngeal HNSCCs (Massarelli *et al.*, 2005, Yu *et al.*, 2007) and has recently been linked with the conversion of a potentially malignant oral lesions to OSCC (Pontes *et al.*, 2009). Akt is known to induce morphological changes associated with EMT, loss of cell-cell adhesion and increased motility and invasion in HNSCC (Larue and Bellacosa, 2005). Oral carcinoma cells of epithelial origin ectopically express a mesenchyme-specific transcription factor (HMGA2) at the invasive front, which has a significant impact on tumour progression and patient survival (Miyazawa *et al.*, 2004). However, the definitive evidence that EMT was induced by Akt was provided by a study in which oral squamous cell carcinoma lines overexpressing activated mutants of Akt were shown to undergo EMT and down-regulate E-cadherin (Grille *et al.*, 2003). Snail and SIP1 exhibit an inverse correlation with E-cadherin expression levels in oral carcinoma cells (Yokoyama *et al.*, 2001, Maeda *et al.*, 2005). The stably Snail overexpressing OSCC clones showed spindle morphology, increased expression of vimentin and decreased expression of E-cadherin (Taki *et al.*, 2003). Julien reported that activation of NF-κB by Akt up regulates Snail expression and induces EMT in OSCC and expression of the NF-κB subunit p65 is enough for EMT induction (Julien *et al.*, 2007). Recently, Bmi1 was found to inhibit Akt inhibitor, PTEN and thus promotion of Akt activity by Bmi1 promotes EMT. Interestingly, Bmi1 depends on Snail for E-cadherin repression. Thus Bmi1 found to be a player in EMT by activation of Akt, stabilisation of Snail and repression of E-cadherin in HNSCC (Song *et al.*, 2009, Smith *et al.*, 2013). Increased

Twist expression is associated with down- regulation of E-cadherin and also may influence the Akt pathway through unclear mechanism in nasopharyngeal carcinoma cells (Zhang *et al.*, 2007). Another study reported that p-Akt inhibition could induce the mesenchymal to epithelial transition (MET) through its interaction with NF-κB and down regulation of Twist in OSCC cells, suggesting that there may be a positive interaction between Twist and p-Akt during the EMT in OSCC (Hong *et al.*, 2009). Moreover, recent work suggests that EMT induced by SDF-1/CXCR4 system might be involved in the lymph node metastasis of OSCC via activation of PI3K/Akt signalling pathway (Onoue *et al.*, 2006).

Work from our laboratory has indicated that PI3 kinase and Akt pathways are essential for migration of fibroblasts in response to added factors such as Migration stimulating factor (MSF), Epidermal growth factor (EGF) and Transforming growth factor alpha (TGFα) (Schor *et al.*, 2003, Ellis *et al.*, 2007, Ellis *et al.*, 2010a). The addition of PI3 kinase inhibitors blocks the migration stimulating activity of MSF and EGF. The data indicates differences in Akt phosphorylation with different growth factors, for example; MSF reduces and EGF and TGFα both increase phosphorylation. The data also reveals that when an Akt inhibitor (Akti 1/2) is added to fibroblasts, in the collagen gel assay, there is a stimulation of cell migration. The data indicate that MSF promoted fibroblast migration, at least in part, by inhibiting Akt activity (Ellis *et al.*, 2010a).

Extensive studies have demonstrated that activation of three Akt isoforms by phosphorylating different residues determines the substrate selectivity and thus

exerts different biological activity in different cell types. Three highly homologous Akt isoforms have opposing functions in different cancer types (Hutchinson *et al.*, 2001, Hutchinson *et al.*, 2004, Irie *et al.*, 2005, Wyszomierski and Yu, 2005, Yoeli-Lerner *et al.*, 2005, Fayard *et al.*, 2011). As Akt is the central signalling node that incorporates membrane, cytoplasmic and nuclear signals regulating cell fate, analysing Akt isoform and cell type specific signalling pathways and targeting them will contribute to personalise targeted cancer therapy.

1.10 Conventional chemotherapy, their limitations and overcome

1.10.1 Conventional chemotherapeutic agents

There are three aims associated with the use of most regularly used chemotherapeutic drugs (Ophardt, 2003):

1. To damage the DNA of the cancer cells.
2. To stop the cell from replicating by inhibiting the synthesis of new DNA.
3. To stop splitting of the original cell into two new cells or to stop mitosis.

The most commonly used chemotherapy agents and their mechanism of actions are illustrated in the Figure 1.33. The chemotherapeutic agents used for advanced HNSCC include platinums (cisplatin and carboplatin), anthracyclines (adriamycin, epirubicin, and pirarubicin), taxanes (paclitaxel and docetaxel) and antimetabolites (e.g., methotrexate and 5- fluorouracil).

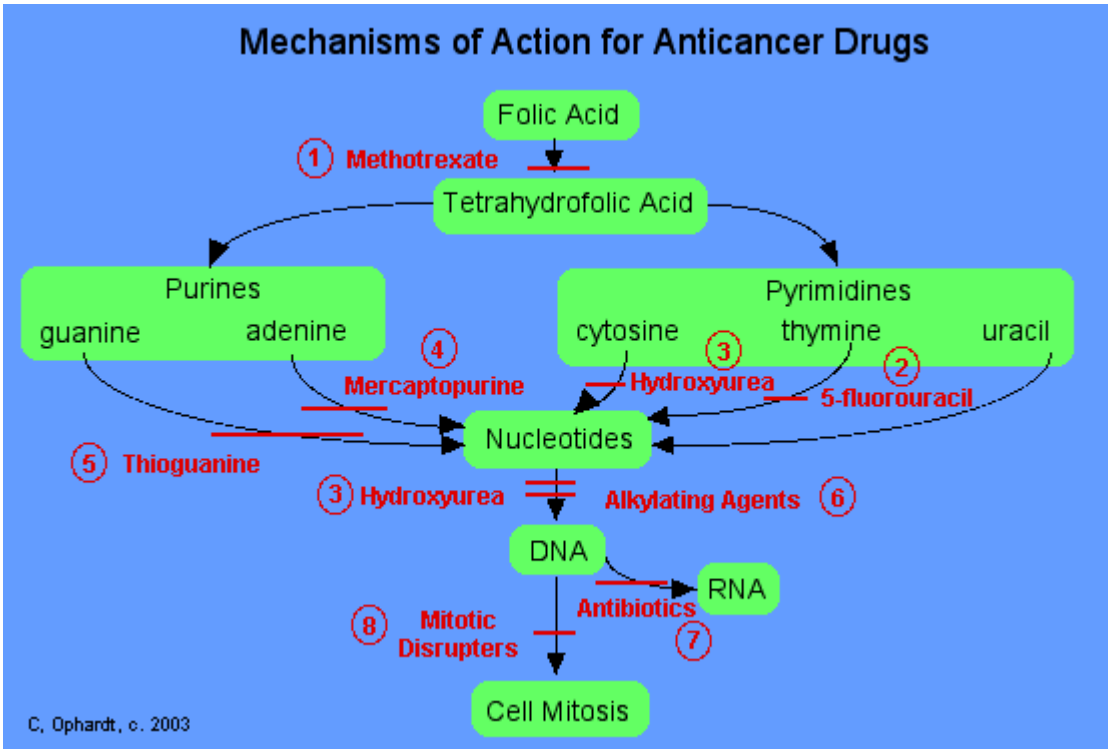


Figure 1.34 Name and mechanism of action of some conventional chemotherapy agents.

Alkylating agents, anti-metabolites, anti-tumour antibiotics, topoisomerase inhibitor and mitotic inhibitors are the most used chemotherapy classes (Ophardt, 2003).

The usefulness of many chemotherapeutic agents is limited by their toxicity to normal cells because there are a very few obvious biochemical differences between cancerous and normal cells. Thus nonspecific actions of conventional anti-cancer agents lead to many side effects. These chemotherapy drugs, whether given alone or in combination, have produced clinical benefits in terms of significant improvement of the overall survival in HNSCC, in comparison to other cancers such as renal and colorectal carcinomas (Lebwohl and Canetta, 1998, Giaccone, 2000, Blackhall *et al.*, 2005). Yet, in most cases, patients unavoidably experience tumor progression or

relapses owing to the development of cells with acquired drug resistance, or appearance of cell subpopulations genetically unmanageable to the drugs (intrinsic drug resistance) (Greenberg *et al.*, 2003).

1.10.2 Mechanisms of chemotherapy drug resistance

The drawbacks of chemotherapy are recognised to be mechanisms that facilitate drug resistance at a cellular level or factors innate to tumour microenvironment and the host. For example, a number of intracellular mechanisms have been associated with reduced drug transport or enhanced efflux including overexpression of plasma membrane efflux proteins such as LRP (lung resistance protein), MRP(MDR-related proteins) and Pgp-170 (P-glycoprotein-170) that inhibit drugs from reaching intracellular targets (Minchinton and Tannock, 2006, Tredan *et al.*, 2007). Mutations in drug target-encoding genes that decrease the attraction of a drug to the active site (e.g. mutations in topoisomerase-coding genes and tubulin that diminish camptothecin and taxanes activity, respectively), improved drug detoxification via upregulation of phase II detoxifying enzymes (e.g. GST, glutathione *S*-transferases), enriched DNA repair mechanisms that neutralise drug-induced DNA damage, along with a variety of other mechanisms that make the cells more resistant to pro-apoptotic signals ranging from cell cycle checkpoints, chromatin and transcriptional modifications and abnormal function of growth factor receptors and tumour suppressor proteins to deregulated intracellular signal transduction pathways (Rudolf and Cervinka, 2003, Dwarakanath *et al.*, 2004).

Tumour microenvironment is a crucial determining factor and transformer of therapeutic response; especially angiogenesis and hypoxia have been broadly studied as options to overcome drug resistance (Folkman, 2007, Maione *et al.*, 2012, Sebens and Schafer, 2012, Semenza, 2012). Even though primary optimism surrounding the targeting of angiogenesis was based on the idea that endothelial cells are genetically stable and therefore less susceptible to develop drug resistance, it is now recognised that tumor blood vessels are unstable and vary from their normal counterparts regarding structural abnormalities in the basement membrane and in pericyte activity, leakiness, morphological characteristics and blood flow (Morikawa *et al.*, 2002, Baluk *et al.*, 2003, Kawamoto *et al.*, 2012). This may explain why tumor endothelial cell become resistant to anti-angiogenic drugs, including the anti-VEGF antibody bevacizumab (Ma and Waxman, 2008).

A recent study revealed that anti-angiogenic agents can decrease the growth of primary tumours, but also can stimulate distant metastases and therefore worsen prognosis. This was mainly because they induced cell invasion signalling, including EMT (epithelial-mesenchymal transition) and hypoxia in tumours. Hypoxia is well-known to stimulate resistance to chemotherapy and radiation. (Braybrooke *et al.*, 2000, Semenza, 2010, Cooke *et al.*, 2012, Semenza, 2012).

Induction of cell heterogeneity and selection of rare cancer stem cell (CSC) variants with intrinsic resistance to chemotherapy are another explanation for chemotherapy failure (da Silva *et al.*, 2012). CSC's are a small subpopulation of tumour cells having tumour- initiating ability and the capability to reconstruct the cellular heterogeneity

of the primary tumour. A number of studies have involved cancer stem cells in cancer progression, invasion process, distant metastasis and loco-regional recurrence after therapy (Prince *et al.*, 2007, Prince and Ailles, 2008, Zhang *et al.*, 2012). Also, it has been reported that cancer cell variants expressing CSC markers are more resistant to chemotherapy than cells that do not express CSC markers (Dean *et al.*, 2005). This suggests that chemotherapy drugs, by targeting the more sensitive non-CSC can contribute to enhancement of the chemotherapy-resistant CSC and therefore stimulate recurrences (Frame and Maitland, 2011).

EMT can regulate cellular plasticity and produce multiple, distinct cellular subpopulations contributing cellular heterogeneity. It also contributes to selection/enhancement of cancer stem cells from well-differentiated epithelial cells (Mani *et al.*, 2008, Polyak and Weinberg, 2009). In this case, well-differentiated cancer cells need to have a higher plasticity and invasive ability via a transformation to non-polarised and poorly differentiated mesenchymal cells (Thiery, 2002, Thiery and Sleeman, 2006). Activation of Wnt/β-catenin, PI3K-Akt, MAPK and Notch signalling pathway can activate EMT and is well-known to occur in OSCC (Boyer *et al.*, 2000, Conacci-Sorrell *et al.*, 2002, Nelson and Nusse, 2004, Larue and Bellacosa, 2005, Nawshad *et al.*, 2005, Barker, 2008, Agarwal *et al.*, 2010, Courtney *et al.*, 2010, Falasca, 2010, Ihle and Powis, 2010). Studies have also revealed a significant correlation between EMT phenotype, drug resistance, and relapses (Zeisberg and Neilson, 2009, Scanlon *et al.*, 2013) including in OSCC patients (Machiels and Schmitz, 2011, Raza *et al.*, 2011).

Figure 1.34 below explains the factors that are responsible for chemotherapy drug resistance.

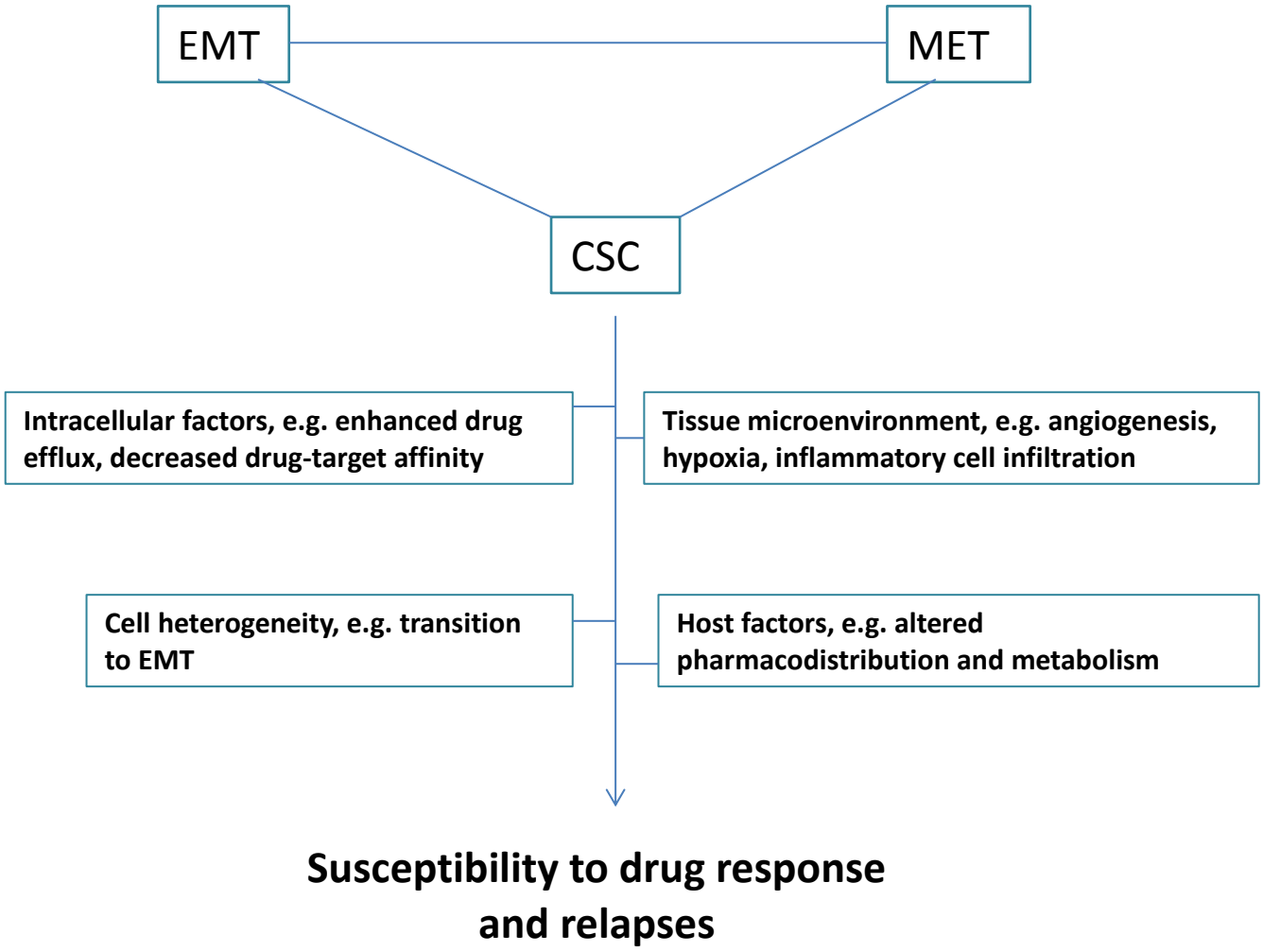


Figure 1.35 Important biological and pharmacological factors implicated in chemotherapy resistance.

Apart from intracellular, tissue micro-environmental and host factors cell heterogeneity plays vital role in drug resistance and relapses (da Silva *et al.*, 2012).

1.10.3 Molecular targeted therapy

Researchers have been working relentlessly to produce drugs that target the mechanism of cancer cells specifically, so as to leave healthy cells undamaged, although certain conventional anti-cancer drugs are quite successful treatments for many forms of cancer. The build-up of knowledge about the specific molecular differences between normal and cancerous cells has allowed for the development of molecular targeted cancer therapy (Li *et al.*, 2014).

Exploiting molecular and cell biology to study carcinogenesis developed a greatly increased understanding of the cellular signalling pathways contributing to tumour growth and survival. The detection of tumour suppressors that can be stopped and oncogenes that can be dysregulated to stimulate tumour cell proliferation, uncovered crucial targets specific to tumour cells. Our knowledge has prospered through basic life science research to show that tumour growth also requires the escape of cell death programs, encouraging replicative immortality, initiating angiogenesis and stimulating invasion and metastasis (Hanahan and Weinberg, 2011). Even though many of these processes are facilitated by genetic alteration within the tumour cells, the non-tumour cells and tumour microenvironment as well as the immune system are also associated in this multifaceted process. These biology-based progressions have strongly expanded the prospective selection of anti-cancer strategies. Furthermore, as well as offering novel targets, they have also opened the gate to molecular-targeted agents that selectively interrupt key signalling pathways and, thus potentially have less nonspecific side-effects (Tsantoulis *et al.*,

2007, Papaspyrou *et al.*, 2011, Rao *et al.*, 2012). Many of these drugs are now in clinical use for specific cancer such as Erlotinib (EGFR-tyrosine kinase inhibitor) for invasive pancreatic and NSCLC (Rosell *et al.*, 2012, Troiani *et al.*, 2012), Bevacizumab (anti-VEGF monoclonal antibody) for metastatic cancer of the kidney, lung and colon (Kerr, 2004), Cetuximab and Trastuzumab (anti-EGFR monoclonal antibody) for colorectal carcinoma and HER-2/neu+ breast cancer, respectively (Chang, 2010, Debucquoy *et al.*, 2010), Imatinib (BCR-Abl inhibitors) for chronic myeloid leukaemia (Waller, 2014) and Vorinostat (the proteasome inhibitor) for cutaneous lymphoma (Lansigan and Foss, 2010). Cetuximab is the only US FDA approved drug for use in locally advanced HNSCC in combination with radiotherapy and as a single agent or in combination with platinum based chemotherapy for recurrent or metastatic HNSCC (Dorsey and Agulnik, 2013).

It is clear that a better knowledge of the molecular and biological profile of HNSCC should accelerate the development of more effective targeted therapies. Existing clinical trials with targeted agents in HNSCC are likely to bring encouraging strategies reducing the risk of tumor recurrence and improving survival of patients with HNSCC.

Chapter 2 Aims and hypothesis

2.1 Aims of the project

The aim of this project was to study the role of VEGF-induced phosphorylated Akt on the migration and prognosis of head and neck cancer. The overall objectives of this project were:

a. To investigate the effect of different concentrations of VEGF and incubation time on the phosphorylation of Akt (pAkt) at Thr 308 and Ser 473 residues in normal and cancer cell lines.
b. To determine the effect of VEGF-induced Akt phosphorylation on the migration of different cell lines.
c. To identify the prognostic significance of VEGF-induced Akt phosphorylation in head and neck squamous cell carcinoma.

The specific studies carried out towards these objectives included:

- VEGF-induced phosphorylation of Akt and PTEN were elucidated by SDS-PAGE and Western blotting in normal keratinocytes (HaCaT), normal oral mucosal fibroblasts (MM1), cells from a dysplastic lesion (PM1), mouth cancer-associated fibroblasts (COM D25), oral adeno-squamous cell carcinoma (TYS) and oral squamous cell carcinoma (TR 146). The effect of PI3K/Akt pathway inhibitors (LY294002 and PI103) was also identified in terms of blocking Akt phosphorylation.
- To investigate the effect of VEGF on cell migration, a number of *in vitro* cell migration and invasion assays were carried out for example, modified

Boyden chamber, 3D collagen gel, wound healing and live cell chemotaxis assay. To determine the different role of pAkt Thr308 and pAkt Ser473 in terms of migration, LY294002 (a specific PI3K inhibitor) and PI103 (dual inhibitor of PI3K and mTOR) were used.

- To elucidate the status of phosphorylation of Akt at both residues, immunohistochemistry was performed using a phospho-site-specific antibody on the collected head and neck cancer tissue samples. A statistically significant association among the patient's behavioural and histopathological characteristics including smoking and alcohol with the Akt phosphorylation was identified. The prognostic significance of pAkt in HNSCC was also determined.

2.2 Hypothesis of the study

- It was hypothesised that VEGF-induced Akt phosphorylation would be dependent on cell type, VEGF concentration and duration of VEGF treatment. It was also hypothesised that PI3K/Akt pathway inhibitors would effectively inhibit Akt phosphorylation regardless of the cell type and PTEN would not be expressed in cancer cells.
- Akt phosphorylated at both residues would be responsible for oral cancer cell migration and PI103, a dual inhibitor of PI3K and mTOR could effectively inhibit that migration.

- It was also hypothesised that Akt phosphorylated at both residues would be found highly activated in VEGF positive HNSCC and would be associated with at least one of the risk factors, if not all. Lymph node metastasis in HNSCC patients would be associated with phosphorylation of Akt at both residues as pAkt would be responsible for head and neck cancer cell migration. pAkt would also be an effective prognostic marker.

Chapter 3 Phosphorylation of Akt and its regulation in different cell lines

3.1 Background

Protein phosphorylation is a post-translational modification in which a covalently bound phosphate group from ATP, is transferred to serine, threonine or tyrosine residue of the protein mediated by protein kinases (O'Connor and Adams, 2010). It is the most common mechanism of controlling protein function and transmitting signals through the cells. Thus phosphorylation plays essential functions in the regulation of a number of cellular processes, including signal transduction pathways, cell cycle, growth, apoptosis and differentiation. It is approximated that 30% of human proteins are the substrates for phosphorylation at some time (Cohen, 2000). Additionally, aberrant protein phosphorylation events are associated with many disease states (Huang, 2010).

Serine, threonine and tyrosine have a nucleophilic hydroxyl (-OH) group that attracts the phosphate group on the universal phosphoryl donor adenosine triphosphate (ATP), causing the transfer of the phosphate group to the amino acid side chain and forming adenosine diphosphate (ADP). This process is mediated by protein kinases and magnesium (Mg+2). Phosphorylation is a reversible process in which kinase can phosphorylate and phosphatases can dephosphorylate the substrates. Thus, these two families of enzymes regulate the dynamic character of phosphorylated proteins in a cell. Phosphorylation causes the conformational changes in the phosphorylated proteins and this change can either activate or deactivate the protein (Figure 3.1). The conformational change can also recruit the

neighbouring proteins that have structurally conserved domains that recognise and bind to phosphomotifs. This recruitment is essential for signal transduction, in which downstream effector proteins are recruited to phosphorylated signalling proteins and initiate the signalling pathway (Johnson and Lewis, 2001).

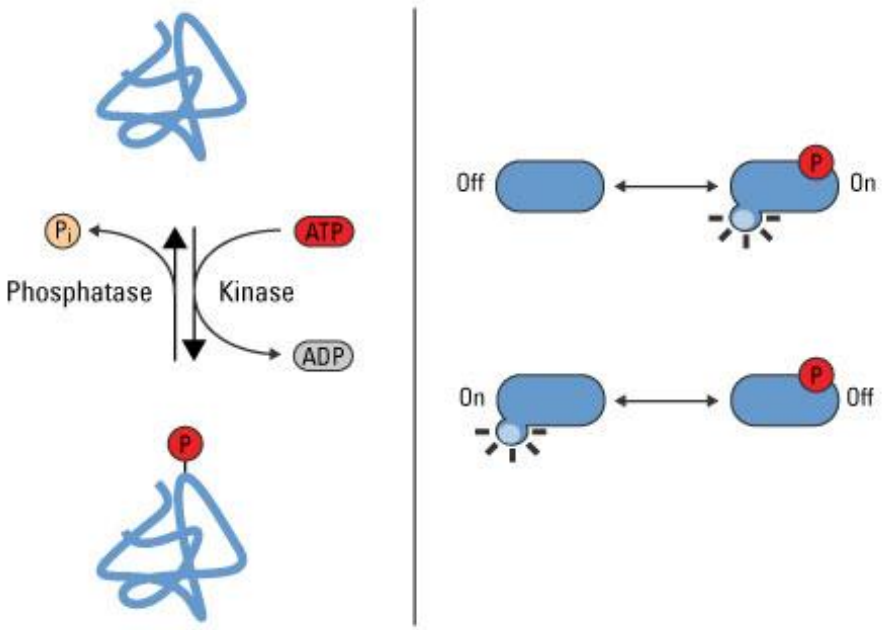


Figure 3.1 Protein phosphorylation.
It is a reversible post-translation modification that controls protein function. Kinases transfers a phosphate group from ATP to the amino acid side chain and phosphatases hydrolyses the phosphate group (left panel). Phosphorylation triggers conformational changes in proteins that either activate (top) or inactivate (bottom) their function (right panel).

Vascular endothelial growth factor (VEGF) mediates its biological effects by binding to specific receptors, leading to receptor dimerisation and following signal transduction by activating PI3kinases . The Class 1 PI3 kinases are a set of lipid kinases that phosphorylate the relatively abundant membrane phospholipid, phosphatidylinositol 4, 5 biphosphate (PIP2), generating small quantities of

phosphatidylinositol 3, 4, 5 triphosphate (PIP3) (Ferrara, 2004). This latter lipid signal controls a diverse set of effector molecules including the Akt group of oncogenic kinases (also known as protein kinase B) (Ellis *et al.*, 2010a). Activation of Akt, a 60 kDa serine/threonine kinase, depends on PI3K (Datta *et al.*, 1999) . Increase of cellular PIP3 by PI3K eventually allows the activation of Akt by phosphorylation at residues T308 (Threonine 308) and S473 (Serine 473) (Alessi and Cohen, 1998). This activation is completed by structural modification stimulated by PI3K-dependent kinase-1 (PDK-1)-dependent phosphorylation at T308 and stabilisation by mTORC2-dependent phosphorylation at S473 (Sarbassov *et al.*, 2005). PTEN ((phosphatase and tensin homologue deleted on chromosome 10) inhibits the activation of Akt by dephosphorylating PIP3.

3.2 Aims and hypothesis

The specific aim of this study was to observe the status of Akt phosphorylation at both T308 and S473 residues in VEGF-treated different cell lines, ranging from normal to cancerous cells. It also aimed to explore the effect of PI3K/Akt pathway inhibitors and the duration of VEGF treatment on the phosphorylation of Akt. PTEN status was also investigated in normal and cancerous cell line.

It was hypothesised that VEGF-induced Akt phosphorylation would be dependent on cell type, VEGF concentration and duration of VEGF treatment. It was also hypothesised that PI3K/Akt pathway inhibitors would effectively inhibit the Akt

phosphorylation regardless of the cell type and PTEN would not be expressed in cancer cells.

3.3 Cell lines

In this study, the following six cell lines were used (Table 9). Validation and justification of using the following cells are described in Appendix 1.

Table 9 Details of the cell lines used

Name	**Cell type**	**Derived/originated from**	**Secondary Source**	**Primary source**
HaCaT	Normal adult keratinocyte	Normal skin	Prof. S. L. Schor (Late), DDS	(Boukamp *et al.*, 1988)
TYS	Oral Adeno-squamous cell carcinoma	Derived from minor salivary gland	Dr. Koji Harada University of Tokushima, Japan	(Yanagawa *et al.*, 1986)
TR 146	Oral squamous cell carcinoma	Originated from buccal mucosa and derived from lymph node	Dr. Sam Crouch, DDS	(Boukamp *et al.*, 1985)
MM1	Normal oral mucosal fibroblast	Normal oral mucosa	-	Dr. M. Macluskey, In-house explant culture of a biopsy, Oral Surgery Clinic, Ninewells
COM D25	Mouth cancer-associated fibroblast	Oral mucosa	-	

				Hospital, Dundee.
PM1	Stromal cell line	Originated from forehead skin and derived from dysplastic lesion	Dr. Sam Crouch, DDS	(Proby *et al.*, 2000)

3.4 Materials

Table 10 lists all the equipment, reagents and antibodies used in this study.

Table 10 List of equipment

Name of the equipment	**Make/Origin**
Class II biological safety cabinet	Medical Air Technology, Manchester, UK
Incubator	Thermo Scientific, Waltham, MA, USA
Water bath	Grant Instruments, Cambridge, UK
Centrifuge machine (Mistral 1000)	MSE, London, UK
Hot Plate	Photax Inc.
Pipette	Thermo Scientific, Waltham, MA, USA
Pipette boy	Integra bioscience, Zizers, Switzerland
Nunclone cell culture dish	Thermo Fisher Scientific, Denmark
Automated cell counter, TC10	Bio-Rad, Hercules, CA, USA
Light Microscope, IX50	Olympus, Tokyo, Japan
Orbital shaker	Stuart Scientific, Staffordshire, UK

Magnetic stirrer	Stuart Scientific, Staffordshire, UK
Mini PROTEAN Tetra System	Bio-Rad, Hercules, CA, USA
Semi-dry transfer cell	Bio-Rad, Hercules, CA, USA
Centrifuge (Sigma 1-13)	Sigma, Osterode am Harz, Germany
Power Pac 300	Bio-Rad, Hercules, CA, USA
ChemiDoc® MP imaging system	Bio-Rad, Hercules, CA, USA

Table 11 List of reagents and antibodies

Cell culture reagents	**Company**	**Catalogue no.**
FCS (Foetal Calf Serum)	Sigma-Aldrich, St. Louis, MO, USA	F-2442
MEM (Minimum Essential Medium Eagle)	Sigma-Aldrich, St. Louis, MO, USA	M-0275
EGTA	Sigma-Aldrich, St. Louis, MO, USA	E-8145
Trypsin	Sigma-Aldrich, St. Louis, MO, USA	T-4549
L-Glutamine	Sigma-Aldrich, St. Louis, MO, USA	G-7513
PBS	Sigma-Aldrich, St. Louis, MO, USA	P-4417
HBSS (HANK'S balanced salt solution)	Sigma-Aldrich, St. Louis, MO, USA	H-4641
Sodium bicarbonate	Merck, Darmstadt, Germany	301515V
DMSO	Sigma-Aldrich, St. Louis, MO, USA	D-5859
Protease inhibitor	Roche Applied Science, Bavaria,	04693116001

	Germany	
Phosphatase inhibitor	Roche Applied Science, Bavaria, Germany	04906845001
SDS-PAGE & WB reagents		
Laemmli loading buffer	Bio-Rad, Hercules, CA, USA	161-0737
2-Mercaptoethanol	Sigma-Aldrich, St. Louis, MO, USA	M-7522
Running buffer (TGS)	Bio-Rad, Hercules, CA, USA	161-0772
Mini-PROTEAN precast gel	Bio-Rad, Hercules, CA, USA	456-1026
Magic Mark XP western std.	Invitrogen, Carlsbad, CA, USA	LC 5602
Immun-Star Western C substrate	Bio-Rad, Hercules, CA, USA	170-5070
Semi-skimmed milk powder	Marvel, London, UK	-
Tris	Boehringer, Mannheim, Germany	708976
Glycine	Sigma-Aldrich, St. Louis, MO, USA	G-8898
SDS	VWR BDH, PA, USA	108073J
Methanol	VWR BDH, PA, USA	101586B
Sodium Chloride	Sigma-Aldrich, St. Louis, MO, USA	S-3014
Hydrochloric acid	VWR BDH, PA, USA	101254H
Tween 20	Sigma-Aldrich, St. Louis, MO, USA	P1379
Nitrocellulose transmembrane	Whatman, GE Healthcare, Buckinghamshire, UK	10401396

Extra thick filter paper	Bio-Rad, Hercules, CA, USA	1703960
Antibodies, proteins and inhibitors		
Phospho-Akt (Ser473)(D9E) XP Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	4060
Phospho-Akt (Thr 308) (C31E5E) Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	2965
Akt (pan) (C67E7) Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	4691
Phospho-PTEN (Ser380) antibody	Cell Signaling Technology Inc., Danvers, MA, USA	9551
Goat anti-rabbit IgG, HRP-linked antibody	Cell Signaling Technology Inc., Danvers, MA, USA	7074
LY294002	Merck Calbiochem, Darmstadt, Germany	9901
PI103	Merck Calbiochem, Darmstadt, Germany	528100
Human Recombinant $VEGF_{121}$	Insight Biotechnology, Middlesex, UK	10-1296
Anti-GAPDH mouse mAb	Millipore, Darmstadt, Germany	MAB374
Rabbit anti-mouse IgG, HRP-linked antibody	Dako, Cambridgeshire, UK	P-0260

3.5 Experimental Procedure

3.5.1 Cell Culture

All the cells were cultured at 37 °C and 5% CO_2 in MEM medium supplemented with 10% (v/v) foetal calf serum (FCS) and 200 mM glutamine. All the cell culture related tasks (including sub-culturing, farming, cryopreservation and resuscitation) were performed according to the standard laboratory protocol prepared by the unit of Cell and Molecular Biology, The Dental School, University of Dundee, UK. The cells were sub-cultured 1-2 time/s a week either 1:3 or 1:4 format. Growth medium was changed every third day. All the cells were grown on 90 mm dishes (Nunclon). To sub-culture, old medium was aspirated off first, washed with 4mls of HBBS, and trypsinised by 2 ml of EGTA at 37^0C for 5 minutes. Floated cells were then neutralised by 2 ml of growth medium and collected in a universal tube. The cell suspension was then spun for 5 minutes at 900 rpm, supernatant was aspirated off and the pellet was re-suspended in growth medium. Cell suspension was then split in required number of dishes and placed in the incubator.

To cryopreserve the cells, steps of sub-culturing were followed up until the re-suspension of the pellets. In this case, pellets were resuspended in freeze mix instead of growth medium and collected in 2 ml cryovials. Vials were then stored in -80^0 C.

To resuscitate the cells, cryovials were first placed in a 37^0 C water bath to defrost. In the meantime, cell culture dishes were prepared by adding 5 ml of growth medium.

Defrosted cells were then placed in the culture dishes containing growth medium and incubated. After 3-4 hours dishes were checked for cell attachment. If cells attached in the dishes, a fresh medium was added. The cell culture log book was maintained in all cases.

3.5.2 Cell treatment and Lysis

Cells were grown on (around 80% confluent) 60 mm culture dishes and then transferred to serum free (SF) medium overnight, prior to the experiment. They were then transferred to the test conditions. Cells were treated with different concentrations of $VEGF_{121}$ (100 pg/ml, 1 ng/ml, 10ng/ml and 100 ng/ml), LY294002 (1 μM and 6 μM) and PI103 (75 nM and 125 nM) for 15 minutes. Cells were also treated with 10 ng/ml of $VEGF_{121}$ for 15 minutes, 5 hours and 24 hours to study the effect of the duration of the treatment on phosphorylation of Akt and PTEN. 5 hours and 24 hours treatment time was selected to compare the status of Akt phosphorylation with cell's migratory behaviour in different migration assays i.e., in the Boyden chamber migration assay (5 hours experiment) and in the scratch and live cell chemotaxis assay (24 hours experiment). Cells were then washed with ice-cold PBS and lysed on ice with RIPA buffer (50 mM Tris HCl, 150 mM NaCl, pH 7.4; 0.1% w/v SDS, 1% v/v Triton x-100, 1% w/v sodium deoxycholate and 5 mM EDTA) containing protease inhibitors for 10 minutes. RIPA buffer containing protease inhibitors with added phosphatase inhibitors was only used to lyse the cells treated

with different concentrations of $VEGF_{121}$ for 15 minutes. Cells were scraped off the dishes after 10 minute and the cell lysates were collected into Eppendorf tubes and frozen at -20^0C.

3.5.3 SDS-PAGE (Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis)

SDS-PAGE was used to separate proteins according to their size. Prior to SDS-PAGE, proteins were denatured by heating at 95^0C in a loading buffer containing SDS and a reducing agent, 2-Mercaptoethanol. Heating in this buffer causes denaturation of the proteins and loss of their 3D structures. SDS, an anionic detergent, binds to the denatured proteins. The amount of bound SDS is relative to the size and proteins therefore migrate according to their size when the current is applied. Protein molecular weight markers were also run on each gel for reference, to allow molecular weight estimation of the fractionated protein. For Western blotting Magicmark XP western standard was used. The separated proteins were transferred to inert nitrocellulose membranes by Western blotting followed by probing the blot with antibodies to identify specific proteins.

The specific procedures of SDS-PAGE are briefly discussed below:

Prior to use in SDS-PAGE cell lysates were thawed and spun at 13,000 rpm for 5 minutes. Samples were then mixed with equal volume of Laemmli sample loading buffer including 5% (v/v) 2-mercaptoethanol. Samples were then heated at 95^0C in a

water bath for 5 minutes and loaded onto 10% or Any kD Biorad TGX precast gels. Gels were then connected to a power pack set at the voltage of 140-180V and run for approximately 40-45 minute. After removing the gels from the cassette they were placed into transfer buffer to be blotted.

3.5.4 Western Blot

Western blot is a method used to transfer the protein bands from SDS-PAGE onto a membrane using an electric current. After transfer, the non-specific binding sites on the blot were blocked by incubation in blocking buffer. The immobilised bands were then probed with specific antibodies against the target proteins (primary antibodies). The primary antibody, which recognises the target protein, was then added. Excess and unbound antibody was removed by thorough washing of the blot. The secondary antibody which recognises the primary antibody was then added and labelled with the enzyme horse radish peroxide for visualisation. The location of this antibody was visualised by adding a substrate to the enzyme HRP generating chemiluminescence.

The procedures of western blotting are briefly discussed below:

After completion of SDS-PAGE, proteins from the gels were electro-transferred onto the nitrocellulose membranes at a constant voltage of 15Vfor 42 minutes. Blots were then blocked by incubation in blocking buffer (1% w/v dried milk in 1X TBST) for 10-30 minutes. Blots were then incubated with the primary antibodies anti-pAkt T308

(1:1000), anti-pAkt S473 (1:2000), anti-pan Akt (1:1000), anti-pPTEN (1: 1000) and anti-GAPDH (1:500) overnight at 19^0C. After washing with TBST blots were then incubated with the secondary antibodies goat anti-rabbit (1:2000) and rabbit anti-mouse (1:10,000) for 60 minute at room temperature. Immunoblots were then developed using a Western C substrate kit and chemiluminescence was detected using a ChemiDoc imaging system.

3.5.5 Statistical Analysis

The data was analysed using the statistical package IBM SPSS 19.0. Differences in the phosphorylation between the negative control and different concentration of $VEGF_{121}$ were carried out using 1-way ANOVA with Bonferroni's post-test. Differences were considered significant when the p value was less than 0.05.

3.6 Results

3.6.1 Akt phosphorylation at S473 (Serine 473) is cell type and VEGF concentration dependent

All the cell lines were treated with different concentrations of $VEGF_{121}$ for 15 minutes and analysed for phosphorylation of Akt at S473 (60 kDa). Phosphorylation was increased in normal oral mucosal fibroblasts (MM1) and OSCC (TR 146) in comparison to the negative control ($p<0.05$) and also with increasing $VEGF_{121}$ concentration (Appendix 2). Phosphorylation was increased in OASCC (TYS) in comparison to the negative control ($p<0.05$) but constant with increasing $VEGF_{121}$

concentration. On the other hand, phosphorylation at S473 was constant in normal keratinocytes (HaCaT) and mouth cancer-associated fibroblasts (COM D25) compared to the negative control ($p>0.05$) and with increasing $VEGF_{121}$ concentration of 100 pg/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml. Akt S473 phosphorylation was either similar or increased compared to that of negative control in all the cell lines except in cells from the dysplastic lesion (PM1). The $VEGF_{121}$-induced AktS473 phosphorylation in PM1 cells was decreased compared to the negative control ($p<0.05$) but increased with different concentrations. The highest increase in Akt phosphorylation at S473 was stimulated by $VEGF_{121}$ concentration of 10 ng/ml in oral cancer cells. Serum free treated cells were used as a negative control and FCS media treated cells were used as a positive control. Figure 3.2 illustrates the status of $VEGF_{121}$-induced Akt phosphorylation at S473 in different cell lines and Table 12 represents the quantification of the phosphorylation.

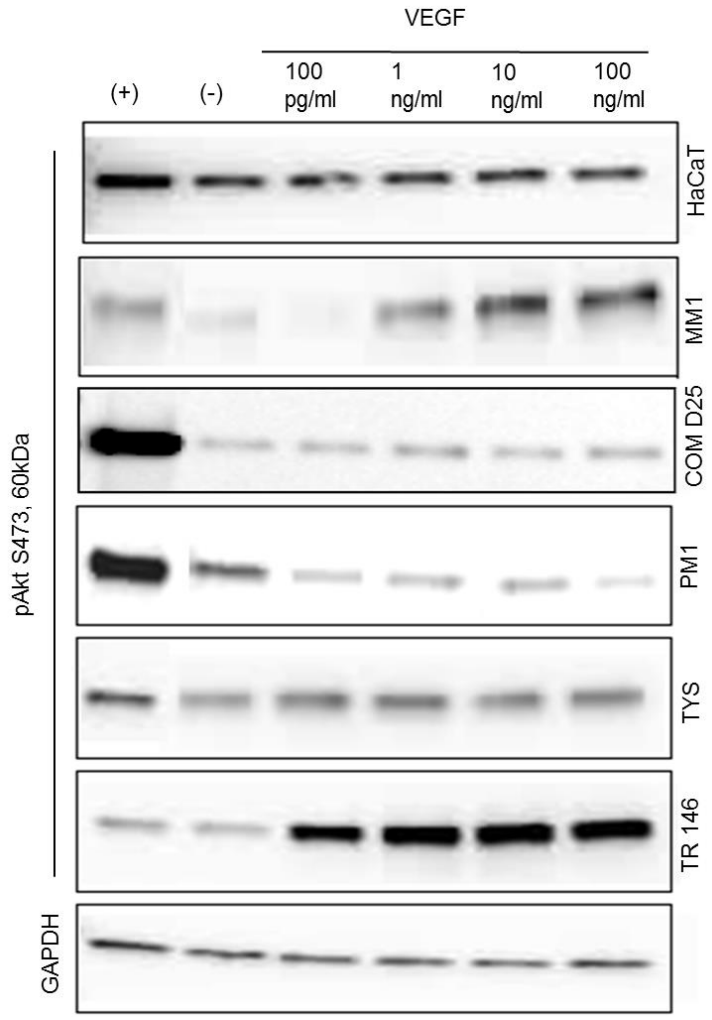


Figure 3.2 Western blot experiments for Akt phosphorylation at Serine 473 in 6 different cell lines with a spectrum of VEGF concentrations.

pAkt S473 phosphorylation in normal keratinocytes (HaCaT), normal oral fibroblasts (MM1), mouth cancer-associated fibroblasts (COM D25), cells from a dysplastic lesion (PM1), OASCC (TYS) and OSCC (TR 146) with VEGF concentrations of 100 pg/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml. All the cells were treated with VEGF for 15 minutes. All the blots were cropped from the original images and the figure shows the representative images from triplicate experiment. (+) denotes the positive control (FCS media) and (−) denotes the negative control (serum free media). GAPDH was used as loading control which showed constant pAkt S473 with all the factors.

Table 12 Quantification of Akt phosphorylation at S473

pAkt Ser473 quantification,%						
Cell name	(-)ve control	(+)ve control	100pg/ml VEGF	1 ng/ml VEGF	10 ng/ml VEGF	100 ng/ml VEGF
HaCaT	100	207*	99	105	103	106
MM1	100	142*	59*	129*	427*	281*
COM D25	100	642*	95	101	79	99
PM1	100	633*	34*	51*	31*	50*
TYS	100	152*	155*	149*	145*	129*
TR146	100	116	451*	632*	678*	600*
GAPDH						
	100	109	104	95	90	106

Note: Each value represents % phosphorylation and (*) denotes significant at $p<0.05$ level compared with negative control.

3.6.2 $VEGF_{121}$-induced pAkt T308 (Threonine 308) is also dependent on cell type and concentration.

pAkt T308 was also analysed in cells after 15 minutes of $VEGF_{121}$ treatment.

Akt phosphorylation at T308 in normal keratinocytes (HaCaT), mouth cancer-associated fibroblasts (COM D25) and OASCC (TYS) was constant in comparison to negative control ($p>0.05$) and with increasing $VEGF_{121}$ concentrations.

Phosphorylation was increased in oral cancer cells (TR 146) (Appendix 2) and decreased in those from a dysplastic lesion (PM1) compared to the negative control ($p<0.05$) and with increasing $VEGF_{121}$ concentration. pAkt T308 was constant at 100 pg/ml and 1 ng/ml $VEGF_{121}$, but increased ($p<0.05$) compared to negative control.

The highest increase in Akt phosphorylation at T308 was at a $VEGF_{121}$ concentration of 10 ng/ml in oral cancer cells. Serum free treated cells were used as negative

control and FCS treated cells were used as a positive control. Figure 3.3 illustrates the status of $VEGF_{121}$-induced Akt phosphorylation at T308 in different cell lines and Table 13 represents the quantification of the phosphorylation.

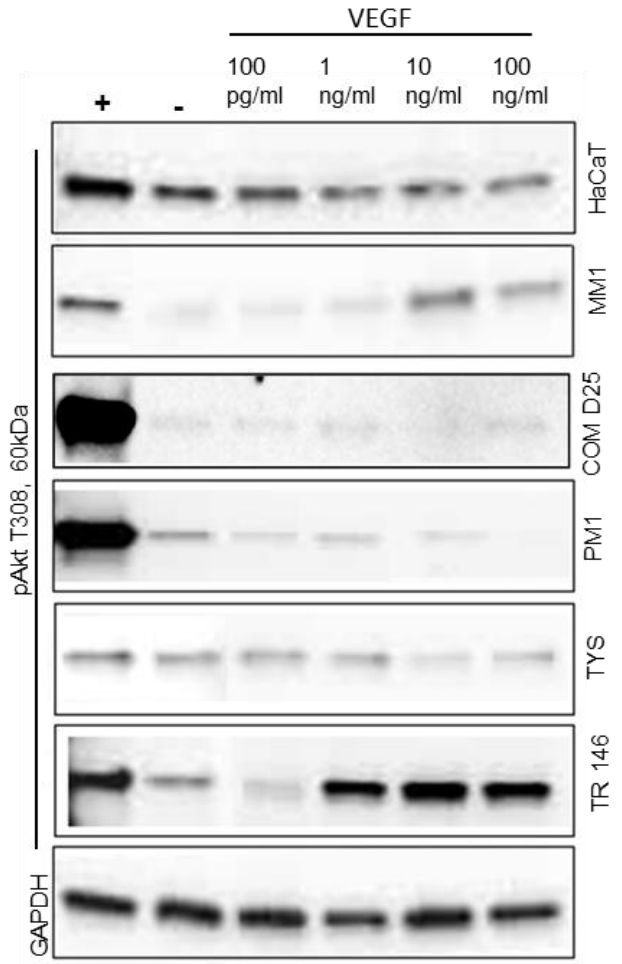


Figure 3.3 Differential phosphorylation of Akt at T308 in different cell lines. $VEGF_{121}$-induced pAkt T308 was dependent on cell type and concentration. All the cells were treated with VEGF for 15 minutes. All the blots were cropped from the original images and the figure shows the representative images from triplicate experiment. (+) denotes the positive control (FCS media) and (−) denotes the negative control (serum free media). GAPDH was used as loading control.

Table 13 Quantification of Akt phosphorylation at T308

pAkt Thr308 quantification, %						
Cell name	(-)ve control	(+)ve control	100pg/ml VEGF	1 ng/ml VEGF	10 ng/ml VEGF	100 ng/ml VEGF
HaCaT	100	215*	102	86	87	82
MM1	100	492*	95	112	486*	455*
COM D25	100	451*	95	100	114	116
PM1	100	1015*	44*	60*	36*	25*
TYS	100	132*	97	113	94	118
TR146	100	180*	155*	771*	1036*	954*
GAPDH						
	100	110	101	92	99	95

Note: Each value represents % phosphorylation and (*) denotes significant at $p<0.05$ level compared with negative control.

3.6.3 Pan Akt is constant in both normal and cancer cells

$VEGF_{121}$-induced pan (total) Akt was constant in both normal keratinocytes (HaCaT) and in OASCC (TYS) compared to the negative control ($p>0.05$) and with different VEGF concentrations. Serum free treated cells were used as negative control and FCS media treated cells were used as positive control. Figure 3.4 illustrates the status of $VEGF_{121}$-induced Pan Akt in different cell lines and Table 14 represents the quantification of the phosphorylation.

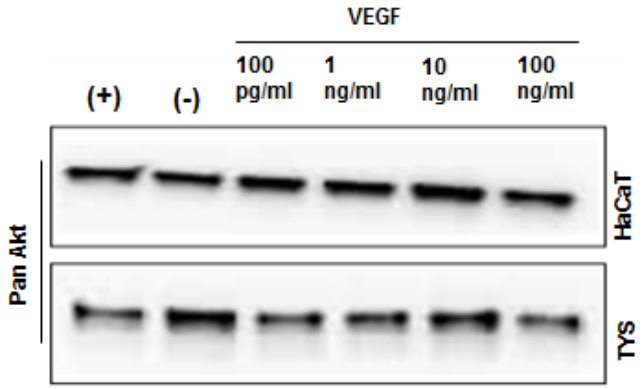


Figure 3.4 Pan Akt status in VEGF121 treated cells.

All the blots were cropped from the original images and the figure shows the representative images from triplicate experiment. (+) denotes the positive control (FCS media) and (−) denotes the negative control (serum free media).

Table 14 Quantification of phosphorylation of pan Akt

Pan Akt quantification, %						
Cell name	(-)ve control	(+)ve control	100pg/ml VEGF	1 ng/ml VEGF	10 ng/ml VEGF	100 ng/ml VEGF
HaCaT	100	108	106	107	112	106
TYS	100	104	91	100	115	89

Note: Each value represents % phosphorylation.

3.6.4 $VEGF_{121}$-induced Akt phosphorylation both at S473 and T308 is time and cell type dependent

Cell lines were treated with 10 ng/ml VEGF for 15 minutes, 5 hours and 24 hours. Western blot experiments show various level of phosphorylation of Akt at both T308 and S473 residues in different cells. Figure 3.5 illustrates the level of Akt phosphorylation in a time-course manner, whereas Table 15 statistically quantifies the level of phosphorylation compared to the respective negative control at different

times. Table 16 compares the VEGF-induced phosphorylation at different times instead of comparing to the respective negative control. Table 16 shows VEGF (10 ng/ml) treatment of COM D25, TYS and TR146 cells (Appendix 3) for various times (15 minutes, 5 hours and 24 hours) caused a gradual increase in the level of Akt phosphorylation at both S473 and T308 ($p<0.05$). By contrast, after 24 h of VEGF exposure either a decrease or a constant level of phosphorylation of Akt at residues T308 and S473 was observed in HaCaT, MM1 and PM1 cells (Figure 3.5) (Table 16).

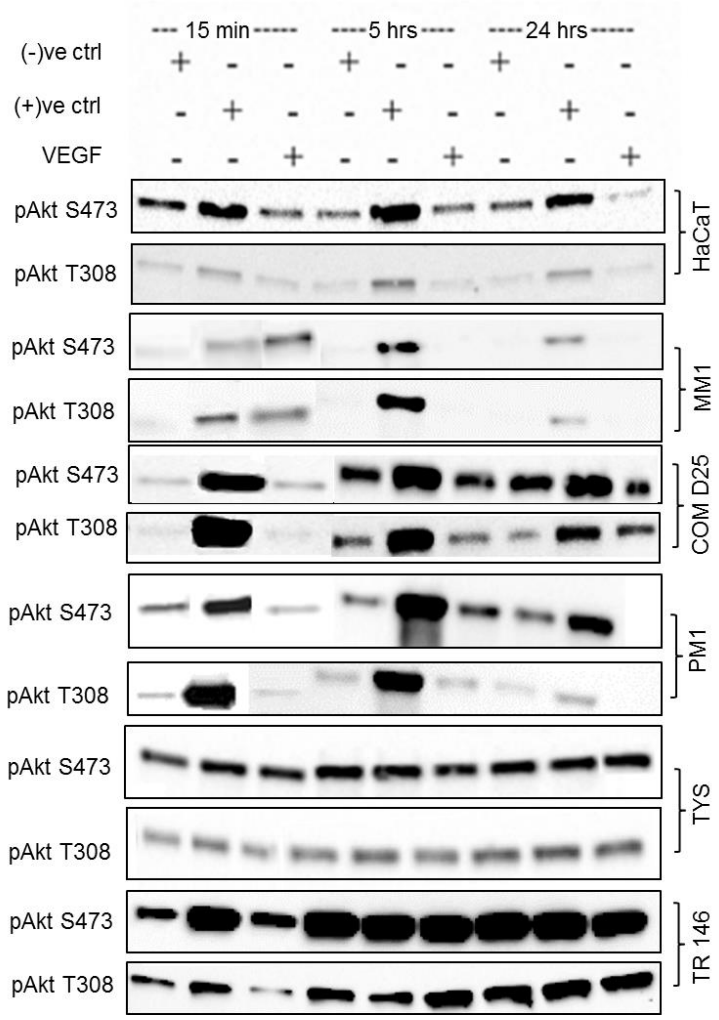


Figure 3.5 Time-course Western blot experiment.

VEGF-induced Akt phosphorylation at both T308 and S473 residues are VEGF incubation time and cell type dependent. All the blots were cropped from the original images and the figure shows the representative images from triplicate experiment. (+) denotes the positive control (FCS media) and (−) denotes the negative control (serum free media).

Table 15 Quantification of Akt phosphorylation at different incubation times

pAkt Ser473 quantification, %									
	15 minutes			**5 hours**			**24 hours**		
Cell name	(-)ve control	(+) control	10 ng/ml VEGF	(-)ve control	(+) control	10 ng/ml VEGF	(-)ve control	(+) control	10 ng/ml VEGF
HaCaT	100	365*	78	100	807*	113	100	209*	27*
MM1	100	142*	427*	100	518*	87	100	383*	99
COMD25	100	589*	103	100	380*	109	100	292*	112
PM1	100	633*	61*	100	830*	182*	100	216*	18*
TYS	100	155*	152*	100	118	92	100	128*	135*
TR146	100	619*	148*	100	92	115	100	108	82
pAkt Thr308 quantification, %									
HaCaT	100	133*	90	100	233*	103	100	146*	91
MM1	100	492*	386*	100	587*	85	100	195*	12*
COMD25	100	982*	98	100	465*	118	100	326*	190*
PM1	100	1015*	85	100	585*	80	100	131*	21*
TYS	100	125*	98	100	126*	103	100	101	113
TR146	100	173*	55*	100	99	130*	100	96	104

Note: Each value represents % phosphorylation. (*) indicates significant changes compared with respective negative control.

Table 16 pAkt quantification compared with VEGF-treatment at different times

Time course, 10ng/ml VEGF-induced pAkt S473 quantification, Mean volume			
Cell name	**15 minutes**	**5 hours**	**24 hours**
HaCaT	781	867	259 (**↓)/ (***↓)
MM1	5825	2059 (*↓)	2047 (***↓)
COM D25	562	2872 (*↑)	2602(***↑)
PM1	13984	28168 (*↑)	1934 (**↓)/ (***↓)
TYS	8261	8185	11049 (**↑)/(***↑)

TR146	9224	43122 (*↑)	36334 (***↑)
Time course, 10ng/ml VEGF-induced pAkt T308 quantification, Mean volume			
HaCaT	4444	4283	3618
MM1	31570	5709 (*↓)	5032 (***↓)
COM D25	480	762 (*↑)	992 (**↑)/(***↑)
PM1	6889	7494	1224 (**↓)/ (***↓)
TYS	5633	6779 (*↑)	9499 (**↑)/(***↑)
TR146	8160	29713 (*↑)	26500 (***↑)

Note: Time-course Western blot experiment revealed that VEGF-induced Akt phosphorylation is time and cell type dependent. Each value represents mean volume. (*) indicates significant changes in phosphorylation compared to 15 minutes, (**) indicates significant changes in phosphorylation compared to 5 hours (***) indicates significant changes in phosphorylation compared to 15 minutes. (↑) indicates increase and (↓) indicates t decrease in phosphorylation.

3.6.5 LY294002 cannot inhibit Akt phosphorylation at S473 in cancer and cancer-associated fibroblast cells

LY2904002 [2-(4-Morpholinyl)-8-phenyl-4 H-1-benzopyran-4-one] is a specific and potent inhibitor of PI3 Kinases, which competitively inhibits ATP binding to the catalytic subunit of the PI3Ks. To elucidate the effects of PI3K inhibitor on the phosphorylation of Akt, cells were treated with LY294002 (1 μM and 6 μM) for 15 minutes. Akt phosphorylation at T308 was effectively blocked by both concentrations of LY294002 in all cells, which was statistically significant ($p<0.05$). Both the concentrations of LY294002 on the other hand, did not block the phosphorylation of Akt at S473 in cancer (Appendix 4)and cancer-associated fibroblast cells either alone or in combination with VEGF ($p>0.05$). pAkt S473 was only blocked by 6 μM LY in normal keratinocytes ($p<0.05$) (Figure 3.6) (Table 17).

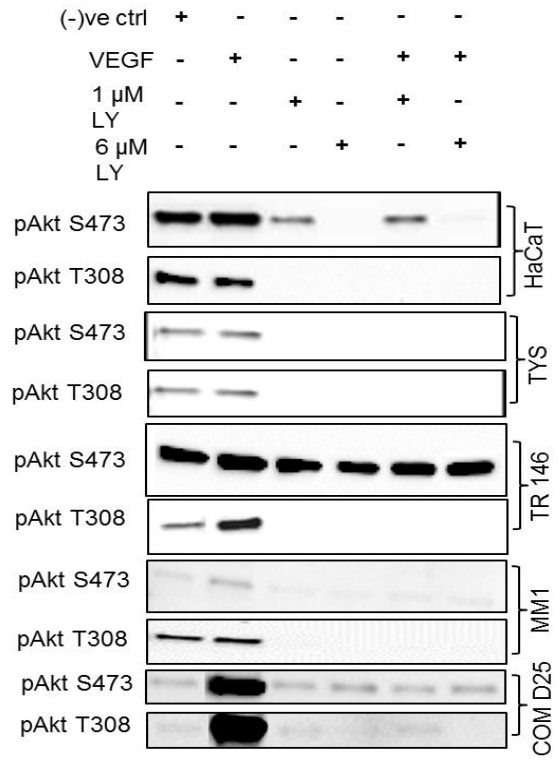


Figure 3.6 Effect of LY294002 on Akt phosphorylation.

pAkt S473 was not blocked by LY in cancer and cancer-associated fibroblast cells. All the blots were cropped from the original image.

Table 17 Quantification of Akt phosphorylation in LY294002 treated cells

pAkt Ser473 quantification, %					
Cell name	VEGF	1 µM LY	6 µM LY	1 µM LY + VEGF	6 µM LY + VEGF
HaCaT	100	25*	2*	32*	1*
TYS	100	2*	2*	4*	1*
TR146	100	112	116	124*	129*
MM1	100	3*	2*	4*	2*
COM D25	100	52*	65*	59*	62*
pAkt Thr308 quantification, %					
HaCaT	100	2*	1*	3*	1*
TYS	100	1*	1*	2*	2*
TR146	100	2*	3*	1*	2*
MM1	100	2*	1*	1*	2*
COM D25	100	1*	2*	1*	1*

Note: Each value represents % changes and (*) denotes significant changes in phosphorylation.

3.6.6 PI103 effectively blocked pAkt at both residues in all cells

PI 103 is a cell permeable pyridinylfuranopyrimidine compound (3-(4-(4-Morpholinyl)pyrido[3′,2′ :4,5]furo[3,2-d]pyrimidin-2-yl)phenol) that acts as a potent and ATP competitive inhibitor of PI3K, mTOR and DNA-PK. Cells were treated with PI103 (75 nM and 125 nM) for 15 minutes. All the cells were effectively and significantly blocked by PI103 ($p<0.05$) with 125 nM being the most effective concentration (Figure 3.7) (Table 18) (Appendix 4).

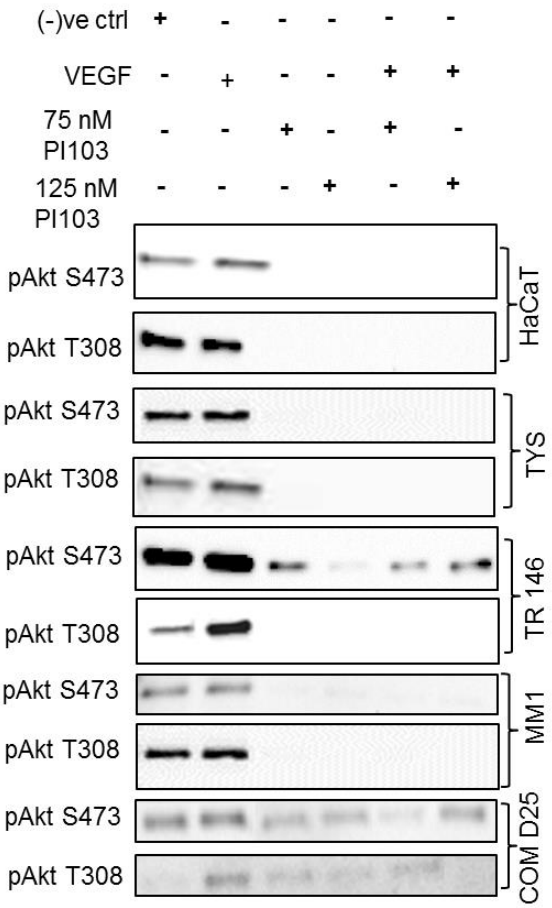


Figure 3.7 Effect of PI103 on Akt phosphorylation.

pAkt was blocked by PI103 in all cells. All the blots were cropped from the original image.

Table 18 Quantification of Akt phosphorylation in PI103 treated cells

pAkt Ser473 quantification, %					
Cell name	VEGF	75 nM PI103	125 nM PI103	75 nM + VEGF	125 nM + VEGF
HaCaT	100	2*	1*	3*	1*
TYS	100	2*	1*	1*	2*
TR146	100	43*	5*	19*	25*
MM1	100	2*	1*	1*	2*
COM D25	100	35*	38*	23*	42*
pAkt Thr308 quantification, %					
HaCaT	100	2*	4*	3*	1*
TYS	100	2*	3*	1*	5*
TR146	100	3*	2*	1*	1*
MM1	100	2*	1*	1*	1*
COM D25	100	19*	15*	25*	10*

Note: Each value represents % changes and (*) denotes significant changes in phosphorylation.

3.6.7 PTEN is inactivated in cancer cells

Phosphatase and Tensin homologue (PTEN) (57 kDa) status was assessed in normal and cancerous cells treated with SF (negative control), FCS (positive control) and VEGF for 15 minutes, 5 hours and 24 hours. Phosphorylated PTEN at S380 residue was constant in normal keratinocytes (HaCaT) and normal oral fibroblasts (MM1) cells over the treatment period. Oral squamous cell carcinoma (TR 146) treated with all the controls and VEGF, expressed very low or no phosphorylated PTEN in different incubation time (Appendix 5). pPTEN at Ser 380 was gradually decreased by increasing incubation time in VEGF-treated mouth-cancer associated fibroblast cells (Figure 3.8).

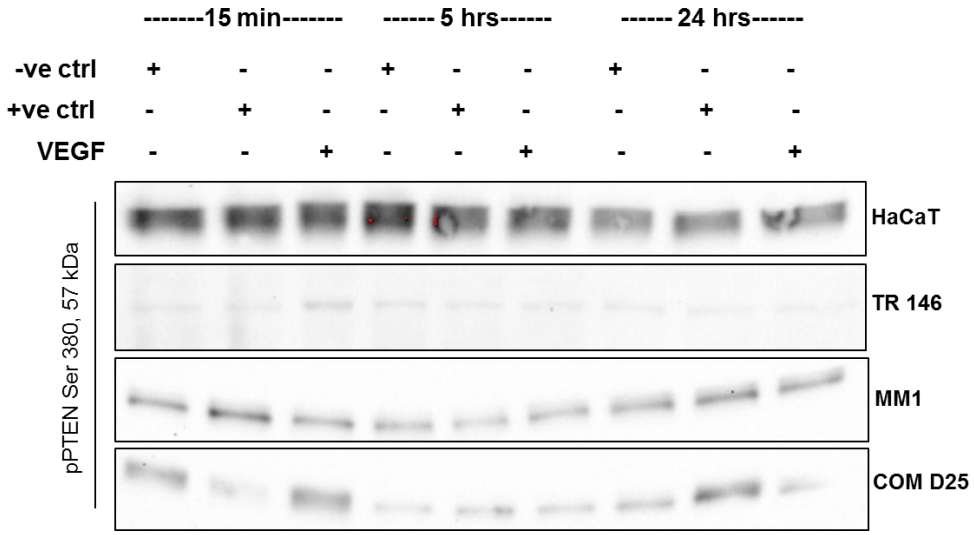


Figure 3.8 Phosphorylated PTEN at S380 in normal and cancerous cells.
pPTEN was inactivated in cancer cells (TR146). All the blots were cropped from the original images. SF treated cell were used as (-)ve control and FCS treated cells were treated with (+)ve control.

3.7 Discussion

To our knowledge, the study of Akt activation by $VEGF_{121}$ in different cell lines ranging from normal epithelial to oral cancer cells as well as normal fibroblasts to oral cancer-associated fibroblasts is the first of its kind. Due to its increased acidity, $VEGF_{121}$ circulates more freely than the other VEGF isoforms, which bind more tightly with vascular heparin sulphate (Ferrara and Davis-Smyth, 1997). $VEGF_{165}$ is the most extensively studied among the other VEGFA isoforms, although $VEGF_{121}$ isoform is also predominant. Exposure of six different cell types to various concentrations of $VEGF_{121}$ resulted in phosphorylation of Akt both at T308 and S473 residues. The phosphorylation at both residues and its intensity and duration

depends on VEGF concentration, duration of exposure and cell type. LY294002, a selective class I PI3K inhibitor is still used extensively in experimental models although it failed in clinical trials due to its poor solubility and high toxicity (Knight, 2011). It inhibited the phosphorylation of Akt at T308 in all the cell lines used in this study. This confirms the highly conserved PI3K-dependent mechanism of Akt phosphorylation at T308. Conversely, LY294002 was able to inhibit the phosphorylation of Akt at S473 only in normal keratinocytes, normal oral mucosal fibroblasts and oral adeno-squamous cell carcinoma, but not in oral cancer and oral cancer-associated fibroblasts. A selective class I PI3K, mTOR (C1 & C2) and DNA-PK inhibitor, PI103, inhibited Akt phosphorylation both at T308 and S473 in all the cell lines. A number of studies have revealed the different mechanisms of Akt phosphorylation at S473. Depending on the context, phosphorylation of Akt at S473 is recognised by a number of different kinases namely, PDK2 (Phosphoinositide-dependent kinase 2) (Chan and Tsichlis, 2001), MAPKAP-K2 (Mitogen-activated protein kinase associated protein kinase 2) (Alessi *et al.*, 1996), ILK1 (Integrin-linked kinase 1)(Lynch *et al.*, 1999) which has been the subject of considerable debate. Sarvassov *et al* in 2005 confirmed that phosphorylation of Akt at S473 in response to growth factors is dependent on Rictor-mTOR complex, mTORC2 (Sarbassov *et al.*, 2005). A number of studies have also proposed that a member of the PI3K-like family of kinases, the DNA-dependent protein kinase (DNA-PK) triggers phosphorylation of Akt at the S473 in response to genotoxic stimuli (Feng *et al.*, 2004, Bozulic *et al.*, 2008). It has also been suggested that Akt can be autophosphorylated at

S473 residue (Toker and Newton, 2000). A recent review by Alex Toker (2014) has suggested that most of these mechanisms are anecdotal and are likely to be cell type-dependent (Toker and Marmiroli, 2014). This theory is proven by this study but the hypothesis is proven incorrect. It has been shown that phosphorylation of Akt at S473 in oral cancer and cancer-associated fibroblast cells depend upon mTORC2 and/or DNA-PK, whereas S473 phosphorylation in normal keratinocytes, normal mucosal fibroblasts and oral adeno-carcinoma was not mTORC2-dependent but PI3K-dependent. Akt phosphorylation is also targeted by dual lipid and protein phosphatase and tensin homologue (PTEN) by dephosphorylating PIP3. Importantly, loss of PTEN expression or inactivating mutations leads to constitutive activation of Akt signalling, characterised by increased cell proliferation and resistance to apoptosis, turning a normal cell to cancerous one (Leslie *et al.*, 2009, Barata, 2011). It has been observed that Akt phosphorylation is higher and there is no PTEN phosphorylation in the oral cancer cell line (TR146) in this study. These characteristics of oral cancer cells here and in other studies with cancer (but not oral cancer) (LoPiccolo *et al.*, 2008, Newton and Trotman, 2014) prompted us to study Akt hyper-phosphorylation and its association with metastasis in the next stage of this project.

Chapter 4 Is there a pAkt between VEGF and motility in oral cancer?

4.1 Background

There has been a significant progress in cancer treatment over the past few decades but oral cancer still has a poor survival rate, with a high occurrence of metastases (Tankere *et al.*, 2000). Cell migration is an essential part of most tumour metastasis. Cells need to migrate away from their microenvironment to enable the tumour to spread or metastasise. Growth factors and matrix macromolecules are essential for the movement of cells (Schor, 1994). Such movement requires a reorganisation of the actin cytoskeleton, which is under the control of many pathways including the PI3 kinase/Akt signal transduction pathway. Previous work in this laboratory showed that the PI3 kinase and Akt pathways are essential for the migration of fibroblasts in response to added factors such as Epidermal growth factor (EGF) and Transforming growth factor alpha (TGFα) (Ellis *et al.*, 2007). The addition of PI3 kinase inhibitors blocks the migration stimulating activity of EGF and TGFα, the data indicating that both growth factors increase phosphorylation of Akt. Inhibition of PI3K activity blocks migration stimulated by G protein-coupled receptors or by receptor tyrosine kinases, signifying that PI3K has a vital function in cell migration (Barber and Welch, 2006). Vascular endothelial growth factor (VEGF) has been reported to stimulate the proliferation of endothelial cells and to enhance vascular permeability and survival (Ferrara *et al.*, 2003). Over-expressed VEGF acts as an effective angiogenic cytokine, stimulating endothelial cells thus promoting angiogenesis in solid tumours such as breast or ovarian carcinomas (Sia *et al.*, 2013). Apart from angiogenic function, VEGF

was also found to be responsible for the migration of breast carcinoma (Bachelder *et al.*, 2003, Mercurio *et al.*, 2005), colon carcinoma (Oommen *et al.*, 2011), hepatocarcinoma (Xu *et al.*, 2012) and skin cancer (Beck *et al.*, 2011). But the role of VEGF and its downstream PI3K/Akt signalling pathway in oral cancer metastasis is not known.

4.2 Aims and Hypothesis

The aim of this study was to establish the role of the PI3K-Akt pathway in $VEGF_{121}$ induced migration of oral cancer cells. It was also aimed to investigate the role of phosphorylated Akt at both T308 and S473 on oral cancer cell migration. A novel class I PI3K, mTOR and DNA-PK inhibitor, PI103 was also used in this study to investigate its inhibitory effect on the migration of oral cancer. The resultant data would help extend the spectrum of known biological activities of this pathway and support the proposal that inhibition of this pathway would be a suitable target for chemotherapeutic drug design to control oral cancer cell metastasis. It would also help to understand why the current used treatments targeting the VEGF pathway in cancer are not universally effective in inhibiting metastasis tumours.

It was hypothesised that Akt phosphorylated at both residues would be responsible for oral cancer cell migration and PI103, a dual inhibitor of PI3K and mTOR would effectively inhibit that migration.

4.3 Materials

The cell lines that were used in this study were as described earlier in Chapter 3 (Table 9). Table 19 and 20 lists all the equipment, reagents and antibodies used in this study.

Table 19 List of equipment

Name of the equipment	**Make/Origin**
Class II biological safety cabinet	Medical Air Technology, Manchester, UK
Incubator	Thermo Scientific, Waltham, MA, USA
Water bath	Grant Instruments, Cambridge, UK
Centrifuge machine (Mistral 1000)	MSE, London, UK
Hot Plate	Photax Inc., UK
Pipette	Thermo Scientific, Waltham, MA, USA
Pipette boy	Integra bioscience, Zizers, Switzerland
Nunclone cell culture dish	Thermo Fisher Scientific, Denmark
Automated cell counter, TC10	Bio-Rad, Hercules, CA, USA
Light Microscope, IX50	Olympus, Tokyo, Japan
Chemotaxis chamber (48 well)	Neuroprobe Inc., Gaithersburg, MD, USA
Membrane filter (8 μm)	Costar, UK
μ-slide Chemotaxis 2D	IBIDI GmbH, Munich, Germany
Heated stage	IBIDI GmbH, Munich, Germany
Inverted fluorescence microscope (IX70)	Olympus, Tokyo, Japan
Immunopen	Dako, Cambridgeshire, UK

Table 20 List of reagents and antibodies

Name	**Company**	**Catalogue no.**
FCS (Foetal Calf Serum)	Sigma-Aldrich, St. Louis, MO, USA	F-2442
MEM (Minimum Essential Medium Eagle)	Sigma-Aldrich, St. Louis, MO, USA	M-0275
EGTA	Sigma-Aldrich, St. Louis, MO, USA	E-8145
Trypsin	Sigma-Aldrich, St. Louis, MO, USA	T-4549
L-Glutamine	Sigma-Aldrich, St. Louis, MO, USA	G-7513
PBS	Sigma-Aldrich, St. Louis, MO, USA	P-4417
HBSS (HANK'S balanced salt solution)	Sigma-Aldrich, St. Louis, MO, USA	H-4641
Sodium bicarbonate	Merck, Darmstadt, Germany	301515V
DMSO	Sigma-Aldrich, St. Louis, MO, USA	D-5859
BSA	Sigma-Aldrich, St. Louis, MO, USA	A-9418
Methanol	VWR BDH, PA, USA	101586B
Tween 20	Sigma-Aldrich, St. Louis, MO, USA	P1379
Mayer's Haematoxylin	Sigma-Aldrich, St. Louis, MO, USA	MHS-32
Gills 3 Haematoxylin	Brunel Microscope, Wiltshire, UK	095903,
Aqueous mounting media	Sigma-Aldrich, St. Louis, MO, USA	M-1289
Native Type 1 Collagen	In-house	
Normal goat serum	Vactor laboratories, Burlingame, CA, USA	S1000
Phospho-Akt (Ser473)(D9E) XP Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	4060
Phospho-Akt (Thr 308)	Cell Signaling Technology Inc.,	2965

(C31E5E) Rabbit mAb	Danvers, MA, USA	
Akt (pan) (C67E7) Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	4691
Anti-Rabbit IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor® 488 Conjugate)	Cell Signaling Technology Inc., Danvers, MA, USA	4412
LY294002	Merck Calbiochem, Darmstadt, Germany	9901
PI103	Merck Calbiochem, Darmstadt, Germany	528100
Human Recombinant $VEGF_{121}$	Insight Biotechnology, Middlesex, UK	10-1296

4.4 Experimental Procedure

4.4.1 Cell Culture

All the cells were cultured at 37 °C and 5% CO_2 in MEM medium supplemented with 10% (v/v) foetal calf serum (FCS) and 200 mM glutamine. All the cell culture related tasks (including sub-culturing, farming, cryopreservation and resuscitation) were performed according to the standard laboratory protocol prepared by the unit of Cell and Molecular Biology, The Dental School, University of Dundee, UK. The cell culture log book was maintained in all cases.

4.4.2 Boyden Chamber Migration Assay

The Boyden chamber assay is based on the chemotaxis mode of cell migration in which cells migrate towards the concentration gradient of a chemoattractant (Chen, 2005).

A 48-well Boyden chamber (Neuroprobe) (Figure 4.1 A) was used for the *in vitro* migration assays as previously described (Ellis *et al.*, 2010b). In brief, cells suspended in serum-free MEM with bovine serum albumin (2μg/ml) (SF-BSA) were seeded into the upper compartment of the chamber. The lower compartment was filled with different concentrations of $VEGF_{121}$ and inhibitors, diluted with SF-BSA. The two compartments were separated by a porous membrane filter (8μm, Costar, UK) coated with type 1 native collagen. The chambers were incubated for 5 hours at 37°C. The filter was then washed twice in PBS, fixed in cold methanol and stained either with Mayer's (Sigma-Aldrich) or Gills 3 (Brunel Microscope) haematoxylin overnight. The cells on the upper surface of the filter were scraped off with a cotton swab. The membrane was then mounted onto a glass slide and examined under bright field illumination at a magnification of x200. Six replicate wells were used per variable. The numbers of migrated cells adherent to the lower surface of the membrane was counted in 3 random fields per well i.e. 18 fields per variable. Data were expressed as mean cell number per field ± SEM. When comparing different variables, results were expressed as a percentage of the controls.

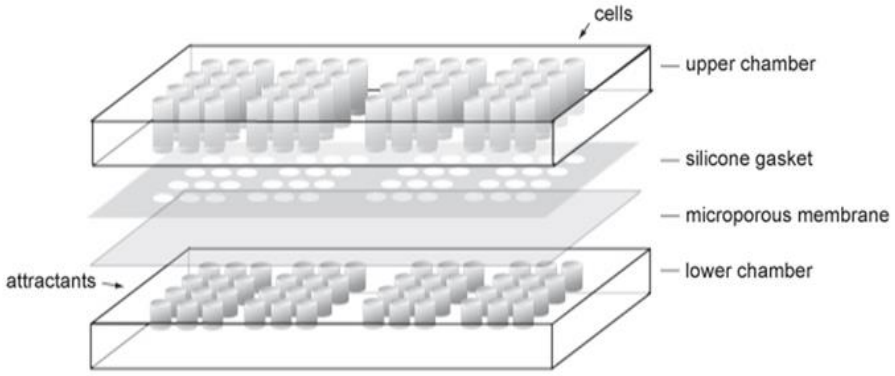
4.4.3 Collagen gel migration assay

The collagen gel migration assay was performed as previously described (Ellis *et al.*, 2010a). Type I collagen from rat tail tendons was used to make 2 ml collagen gels in 35 mm plastic tissue culture dishes as described earlier (Schor *et al.*, 1980). Collagen gels were overlaid with 1 ml of either serum-free MEM (SF-MEM) or SF-MEM containing 4× the final concentration of $VEGF_{121}$ and/or with the inhibitors. Confluent stock cultures of cells were then harvested, resuspended in growth medium containing 4% (v/v) FCS at the desired concentration and 1 ml aliquots were added to the overlaid gels. Considering the 2 ml volume of gel, 1 ml medium overlay and 1 ml cell inoculum, this procedure gives a final concentration of 1% (v/v) serum in both control and test cultures. Cells attached to the surface of the gel within 1 hour and started to migrate into the underlying 3D gel within 24 hours. Four days after plating, the number of cells remaining on the surface or that had migrated into the gel were determined by microscopic observation of 10 randomly selected fields in each of the duplicate cultures. Cell migration was expressed by the number of cells that migrated into the 3D gel, as a percentage of the total number of cells present (Mean ± SEM). When comparing different variables, results were expressed as a percentage of the controls.

4.4.4 Live cell chemotaxis assay

Live cell imaging during chemotaxis was captured while cells were in the μ-slide (IBIDI) (Figure 4.1B). The μ-Slide Chemotaxis 2D is a tool for investigating chemotaxis of adherent migrating cells in 2D over extended periods of time. The linear concentration profile which is necessary for chemotactical movement is produced by diffusion and stable for at least 48 hours. Detailed experimental procedures are described in Appendix 6 and 7. In brief, live cell migration was observed for 24 hours in a heated stage fitted onto an inverted microscope (Olympus IX70). Cells (HaCaT and TR146) were plated in the observation area of the μ-slide chemotaxis 2D (IBI-treat) (IBIDI GmbH) with the control in one reservoir and chemo-attractant with or without the inhibitors in the other reservoir. Time-lapse images were captured every 15 minutes by Metamorph v6.1 software (Molecular Devices). Cells were tracked by the manual tracking plug-in of ImageJ software and analysed by different chemotaxis parameters e.g. centre of mass, forward migration index (FMI) and directness by IBIDI's Chemotaxis and Migration tool. Video of live cell migration and animation movie was also prepared using the ImageJ software (See attached CD).

A



B

Figure 4.1 Equipment for migration assay.

(A) Boyden chamber (B) μ-slide chemotaxis 2D

4.4.5 Wound healing (Scratch assay) and Immunofluorescence assay

The scratch assay, a directional *in vitro* 2D migration assay, was performed as described earlier (Rodriguez *et al.*, 2005). A cell monolayer was serum starved overnight and then a wound was made in the monolayer using a 100μl pipette tip, the assays were then incubated in test conditions (VEGF ± inhibitors) for 24 hours. Images were captured at the starting point and at regular periods throughout the assay to monitor the cell migration causing wound closure. After 24 hours, the cells

were fixed with cold methanol for 15 minutes and then washed with PBS. Cells were then treated with 0.2% Triton X-100 in PBS for 5 minutes, the area into which cells migrated was ringed with Immunopen (DAKO) and blocked with 5% (v/v) normal goat serum (NGS) (Vector Lab) in PBST (phosphate buffered solution with 0.1% Tween 20) for 30 minutes. The cells were then washed with PBS and incubated with pAkt S473 or pAkt T308 diluted in 5% (v/v) NGS in PBST at 4°C for overnight. They were then washed twice with PBST, once with PBS and incubated with secondary antibody conjugated with Alexa Fluor 488 (1:1000) for 30 minutes at room temperature. After washing twice with PBST and once with PBS, sections were cover slipped with aqueous mounting medium (Sigma). Sections were then viewed with an Olympus IX70 inverted fluorescent microscope using x10 or x40 objective lenses. Images were collected using an Olympus SC35 digital camera. All devices were controlled through Metamorph v6.1 software (Molecular Devices) and images were then processed and analysed by ImageJ software (NIH).

4.4.6 Statistical Analysis

The data was analysed using the statistical package IBM SPSS 19.0. Differences in cell migration were analysed by Kruskal-Wallis test and Bonferroni with Dunn's post-test. Differences were considered significant when the *p* value was less than 0.05. The Rayleigh test was applied for unimodal clustering of directions in live cell chemotaxis assay and a $p<0.05$ was chosen as the criterion for rejecting the null hypothesis of random directionality.

4.5 Results

4.5.1 VEGF can stimulate oral adeno-squamous cancer and cancer-associated fibroblast cell migration and can be blocked by LY294002 and PI103

Cell migration experiments were performed using a modified Boyden chamber assay. Different concentrations of VEGF were used to investigate the role of this growth factor in the migration of normal adult keratinocytes (HaCaT), normal oral mucosal fibroblasts (MM1), mouth cancer-associated fibroblast (COM D25) and OASCC (TYS). HaCaT (Appendix 8) and MM1cells were not stimulated to migrate in response to VEGF (Figure 4.2 A, B). However, VEGF stimulated the migration of TYS and COM D25 cells (Figure 4.2 C, D) and this migration displayed a dose response effect with maximal stimulation at approximately 10 ng/ml VEGF ($p<0.05$) (Appendix 9 & 10). A cell permeable, potent, reversible and specific PI3K inhibitor, LY294002, which acts on the ATP binding site of the enzyme, had no effect on the migration of these cells either alone or in combination with VEGF (Figure 4.2 C, D). A blocking effect of LY294002 was observed at concentrations between 1μM to 6μM. No effect on the migration of MM1 and HaCaT was observed in response to LY294002 alone or in combination with VEGF (Figure 4.2 A, B). PI103, another potent, cell-permeable, ATP-competitive PI3K and mTORC1/2 inhibitor was added at 75 nM-250 nM and its effect on cell migration was observed. PI103 reduced HaCaT, MM1 and TYS (Appendix 11) cell migration from baseline to below the baseline level ($p<0.05$) and showed no effect in combination with VEGF (Figure 4.2

A, B, C). PI103 alone had no effect and stimulated COM D25 cell migration in combination with VEGF (Figure 4.2 D). A representative statistical report is attached in Appendix 12.

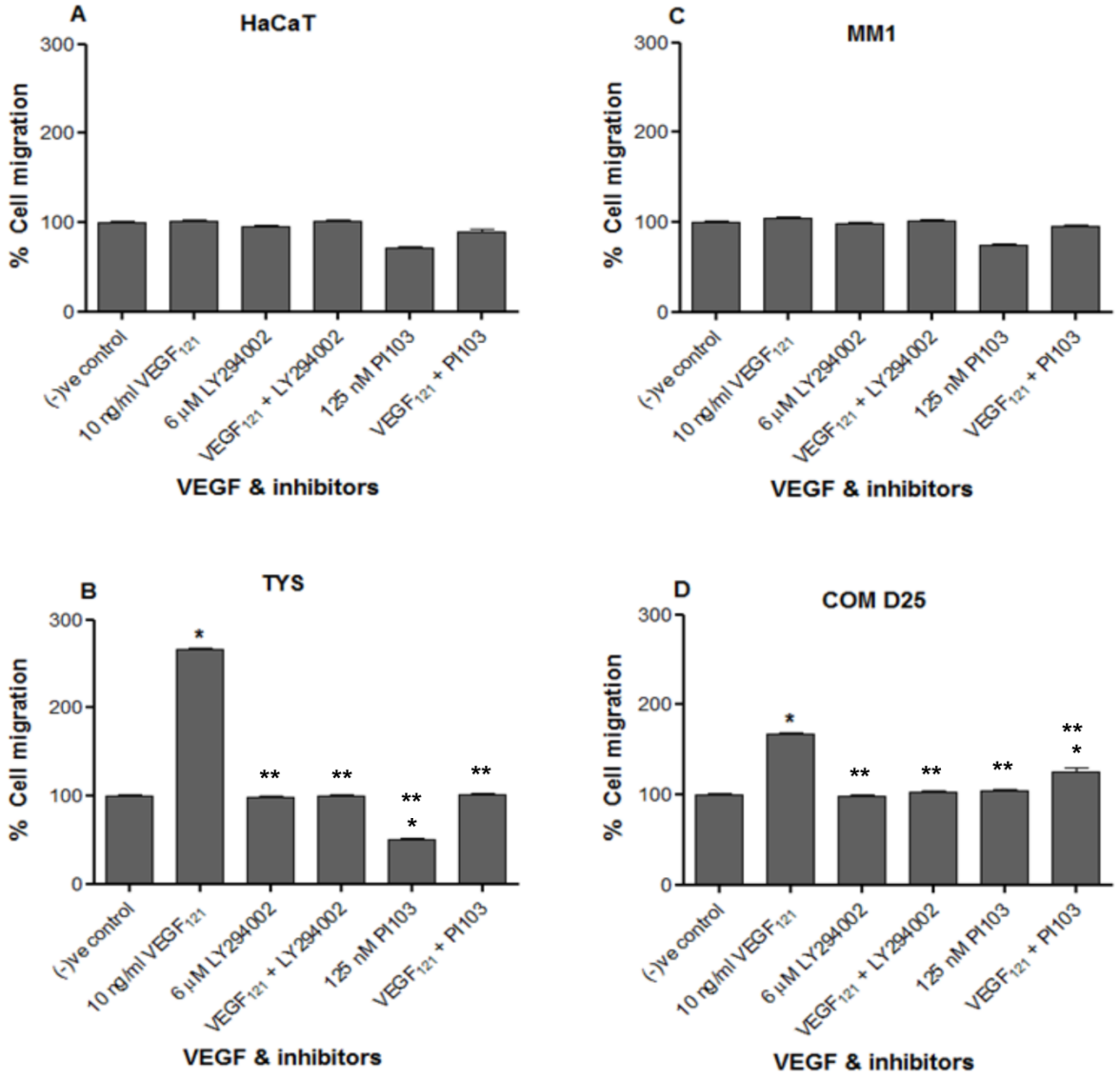


Figure 4.2 Boyden chamber migration assay.

Variable migratory responses of different cell lines treated with VEGF and PI3K-Akt pathway inhibitors obtained by Boyden chamber migration assay compared with negative control. (A) Normal keratinocytes (HaCaT) (n=35) and (B) Oral mucosal

fibroblasts (MM1) (n=40) were not stimulated to migrate by VEGF (baseline, n =32 & 38, respectively) ($p>0.05$). LY294002 had no effect either alone or in combination with VEGF on the migration of HaCaT (n=30 & 33, respectively) and MM1 cells (n=37 & 38, respectively). PI103 alone inhibits the migration of HaCaT (n=10) and MM1 (n=21) to below the baseline (baseline, n=14 & 21, respectively) and has no effect in combination with VEGF (n=13 & 27, respectively). (C) OASCC (TYS) (n=90) and (D) Mouth cancer-associated fibroblast (COM D25) (n=50) were stimulated to migrate through the filters by VEGF (baseline, n=34 & 30, respectively) ($p<0.05$). There is no effect of LY294002 alone or in combination with VEGF on TYS (n= 35 & 34, respectively) and COM D25 (n= 29 & 32, respectively) on cell migration. PI103 alone reduced TYS (n= 13) cell migration from baseline to below the baseline (baseline, n= 24) and showed no effect in combination with VEGF (n=25). However, PI103 had no effect alone on COM D25 cell migration (n=26) and stimulated migration (n=31) ($p<0.05$) in combination with VEGF (baseline, n=25). n= number of cells migrated. Asterisk (*) indicates the significant changes compared with negative control. Double asterisk (**) indicates the significant changes compared with VEGF.

4.5.2 VEGF can stimulate oral cancer cells and cells from a dysplastic lesion to migrate and can be blocked by PI103

Some of the cells lines (TR 146 and PM1) investigated here did not migrate in the modified Boyden chamber assay and therefore, a 3D collagen gel migration assay, developed in other studies, was used. After initial experiments to determine suitable concentrations of VEGF and the Akt inhibitors, the following concentrations were used in the collagen gel assay: 10 ng/ml $VEGF_{121}$, 6 μM LY294002 and 125 nM PI103. Normal oral fibroblasts (MM1) were not stimulated to migrate in response to VEGF

(Figure 4.3 A), whereas VEGF stimulated the migration of cancer-associated fibroblasts (COM D25) (Figure 4.3 B, $p<0.05$), cells from a dysplastic lesion (PM1) (Figure 4.3 C, $p<0.05$) and OSCC (TR146) (Figure 4.3 D, $p<0.05$) after 4 days of treatment.

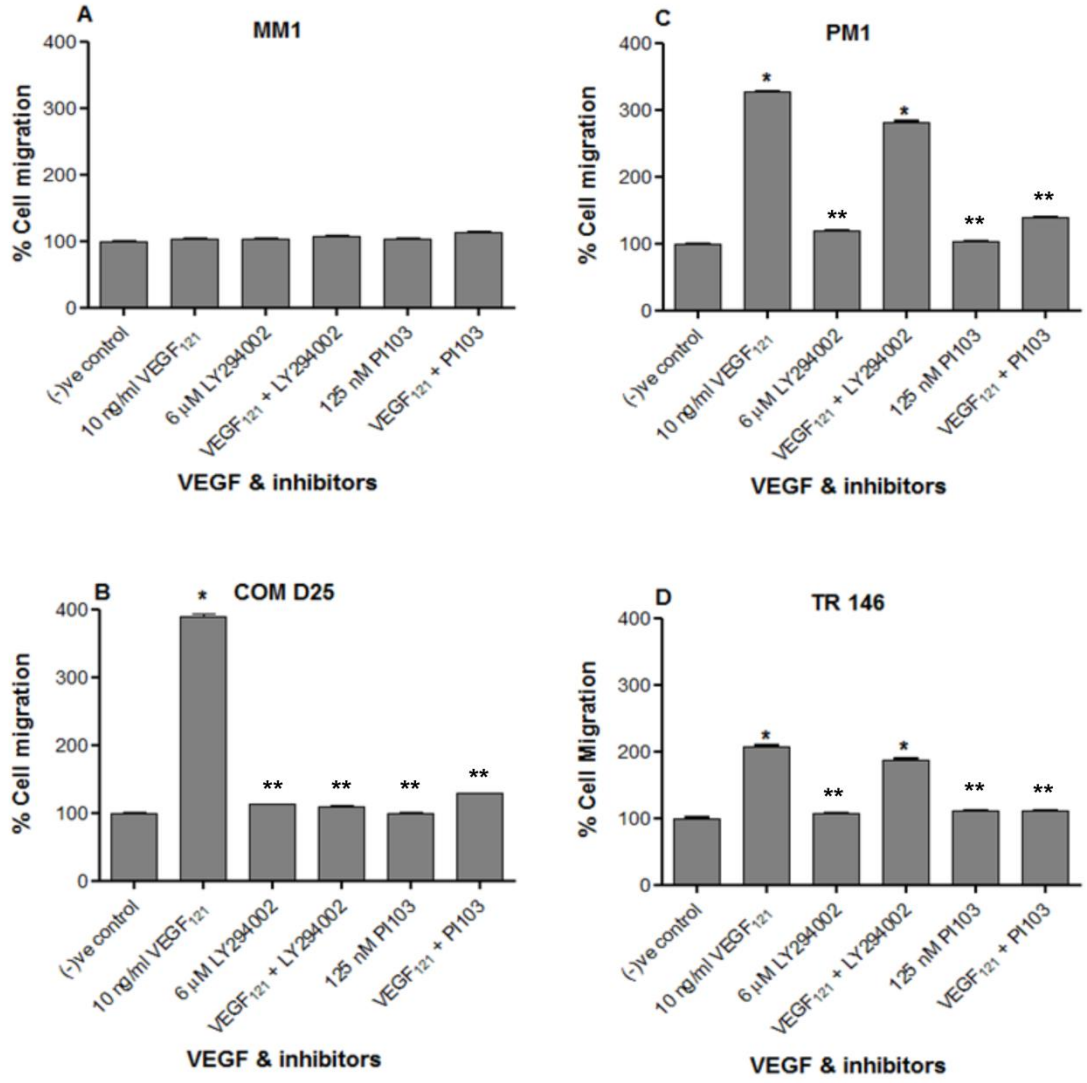


Figure 4.3 3D Collagen gel assay.

VEGF stimulated cells from a dysplastic lesion and oral cancer cells to migrate in the collagen gel assay compared with the negative control. (A) Normal oral mucosal fibroblasts (MM1) were not stimulated to migrate into the collagen gel in response to VEGF and the inhibitors (baseline, n =30) ($p>0.05$). (B) Mouth cancer-associated

fibroblasts (COM D25) were stimulated to migrate (n=33) significantly by VEGF (baseline, n= 8) ($p<0.05$). There was no effect of LY294002 and PI103 alone (n= 9 & 8, respectively) or in combination with VEGF (n=9 & 10, respectively) on COM D25 cell migration. (C) PM1 were stimulated to migrate by VEGF alone (n=7) and in combination with LY294002 (n=6) ($p<0.05$) (baseline, n=2), and no effect with LY294002 & PI103. (D) TR146 were also stimulated to migrate by VEGF alone (n=15) and in combination with LY294002 (n=13) ($p<0.05$) (baseline, n=7), and LY294002 and PI103 had no effect. n= number of cells migrated. Asterisk (*) indicates the significant changes compared with negative control. Double asterisk (**) indicates the significant changes compared with VEGF.

LY294002 and PI103 alone and in combination with VEGF had no effect on the MM1 and COM D25 cell migration (Figure 4.3 A, B). These two inhibitors alone also had no effect on the migration of PM1 (Figure 4.3 C) and TR 146 cells (Figure 4.3 D). LY294002, not PI103, in combination with VEGF stimulated the migration of these cells ($p<0.05$).

4.5.3 Oral cancer cells showed directive cell migration towards VEGF and can be blocked by PI103

Trajectory plots were developed after taking images of a 24 hour period of migration of HaCaT and TR146 cells (Figure 4.4). The plots were showed the directive oral cancer (TR146) cell migration towards VEGF (Figure 4.4 B) (Video clip 4.1 and 4.2) unlike the HaCaT which showed no migration (Figure 4.4 A) (Video clip 4.3 and 4.4)

or TR146 with inhibitors (Video clip 4.5-4.8) which showed random migration (Figure 4.4 B, C). Legends of the video clips are described in the Appendix 13.

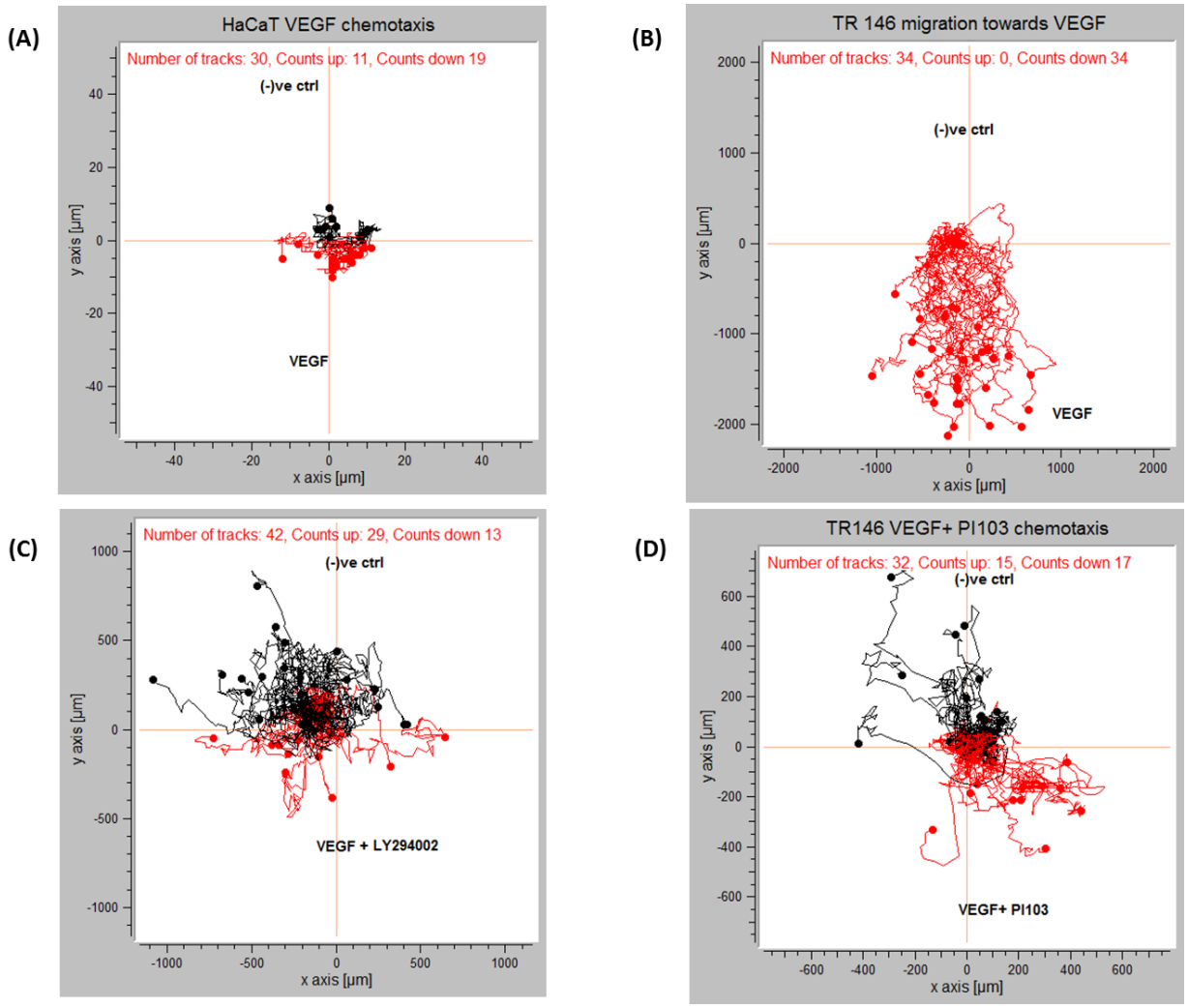


Figure 4.4 Trajectory plot of the live cell chemotaxis assay.

(A) HaCaT cell showed no migration (B) OSCC showed directional migration towards VEGF (C) and (D) OSCC cells with the inhibitors showed random and inhibited migration.

The centre of mass and forward migration index was higher towards chemoattractants (VEGF) than that towards negative control for OSCC. This suggested the directive cell migration towards VEGF which is significant ($P<0.001$)

(Table 21). However, HaCaT cells showed insignificant migration towards VEGF (P>0.05) and PI103 effectively blocks OSCC cell migration (P>0.05). Although LY294002 blocks VEGF-induced OSCC cell migration, it rather showed higher migration towards negative control (P<0.05).

Table 21 Chemotaxis parameter of live cell chemotaxis assay

Chemotaxis parameters	**HaCaT**	**TR 146**		
	-ve vs VEGF	-ve vs VEGF	-ve vs VEGF+LY	-ve vs VEGF+PI
Centre of mass (μm)	X = 2.88	X= -84.55	X=-250.59	X= 60.31
	Y =-1.41	Y= -1379.85	Y=132.62	Y= 34.875
xFMI or FMI I	0.087	-0.024	-0.082	0.026
yFMI or FMI II	-0.051	-0.381	0.039	0.006
Directness	0.257	0.399	0.140	0.102
Rayleigh test	P>0.05	P<0.001	P<0.05	P>0.05

Note: FMI-Forward migration index, xFMI- a direction perpendicular to the gradient, yFMI- a direction parallel to the gradient

4.5.4 pAkt Thr308 was strongly expressed and localised in the nucleus of serum and VEGF-induced migrated cells

24 hour observation of cell migration in the scratch assay (Figure 4.5) showed that VEGF stimulated the migration of cancer-associated fibroblasts, cells from dysplastic lesion and oral cancer cells, whereas normal fibroblasts and keratinocytes were not stimulated to migrate in response to VEGF. LY294002 cannot block the VEGF-induced migration of oral cancer cells in this assay and upon addition of VEGF, cancer cells were stimulated to migrate significantly. On the other hand, LY294002

blocked the migration of cells from dysplastic lesion and this migration can be rescued upon addition of VEGF. No migration of oral fibroblast, cancer-associated fibroblasts and keratinocytes were observed in response to LY294002 alone or in combination with VEGF. PI103 blocks VEGF-induced migration of oral cancer cells insignificantly, whereas these cells were stimulated to migrate upon addition of VEGF. No effect on the migration of oral fibroblasts, cancer-associated fibroblasts, keratinocytes and dysplastic cells was observed in response to PI103 alone and in combination with VEGF. Serum-free MEM was used as negative control, 10% FCS was used as positive control and 10ng/ml VEGF as a stimulant in this study.

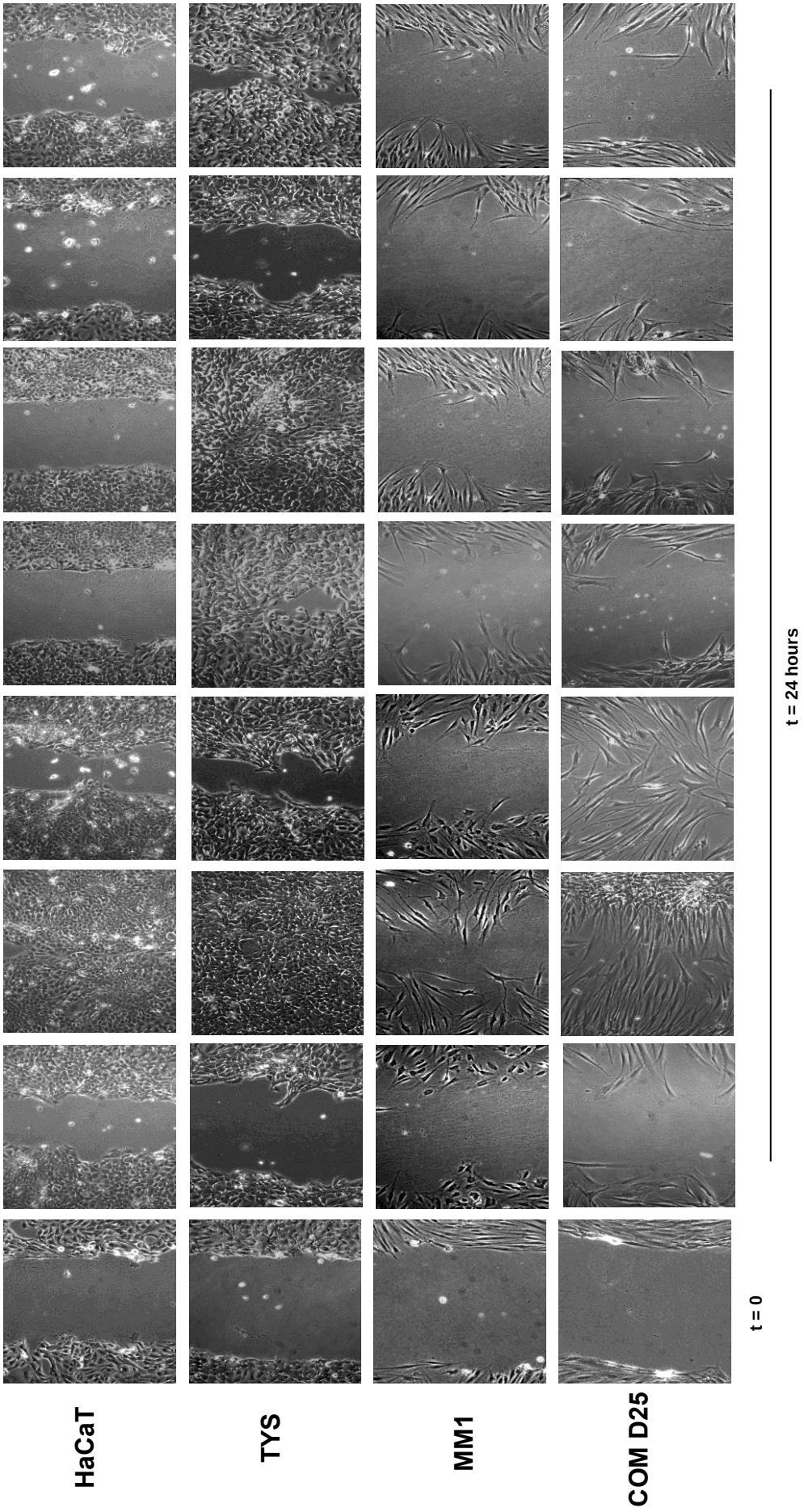

HaCaT
TYS
MM1
COM D25
t = 0
t = 24 hours
(-)ve cntrl
(+)ve cntrl
VEGF
LY294002
VEGF+ LY294002
PI103
VEGF+ PI103

Fig 4.5

Figure 4.5 Scratch assay.

Images have shown variable migratory behaviour of different cell lines. Scratch assays of keratinocytes and normal fibroblasts showed no migratory response upon treatment with VEGF and/or PI3K-Akt pathway inhibitors. Whereas cancer-associated fibroblasts, cells from a dysplastic site and oral cancer cells were stimulated to migrate in response to VEGF. Oral cancer cells showed migration even in serum-free condition and in response to the inhibitors alone or in combination with VEGF. All the images were taken at X100 magnification.

After 24 hours of scratch assay observation, immunocytochemistry of the fixed cells showed the expression and localisation of phosphorylated Akt in the cells. Serum-induced migrated cells of all the cell lines assayed showed strong expression and nuclear localisation of pAkt T308 (Figure 4.6 B), whereas a very weak or diffused expression of pAkt S473 was observed in these migrated cells (Figure 4.6 A). VEGF-induced migration of cancer- associated fibroblasts, dysplastic and oral cancer cells also showed strong expression and nuclear staining for pAkt T308 (Figure 4.6 B) and very weak or diffused staining for pAkt S473 (Figure 4.6 A). LY294002 alone or in combination with VEGF effectively blocks the expression of pAkt S473 in the keratinocytes, normal fibroblasts, cancer-associated fibroblasts and dysplastic lesion cells and showed diffused expression in the cancer cells (Figure 4.6 A). On the other hand, pAkt T308 expression was found to be effectively blocked by LY294002 alone and in combination with VEGF in the keratinocytes, normal fibroblasts and cancer-associated fibroblasts cells and showed nuclear staining in the dysplastic and cancer cells in combination with VEGF (Figure 4.6 B). Both pAkt S473 and pAkt T308 were

found to be expressed very weakly or diffusely by PI103 alone and in combination with VEGF in all the cells (Figure 4.6 A & B).

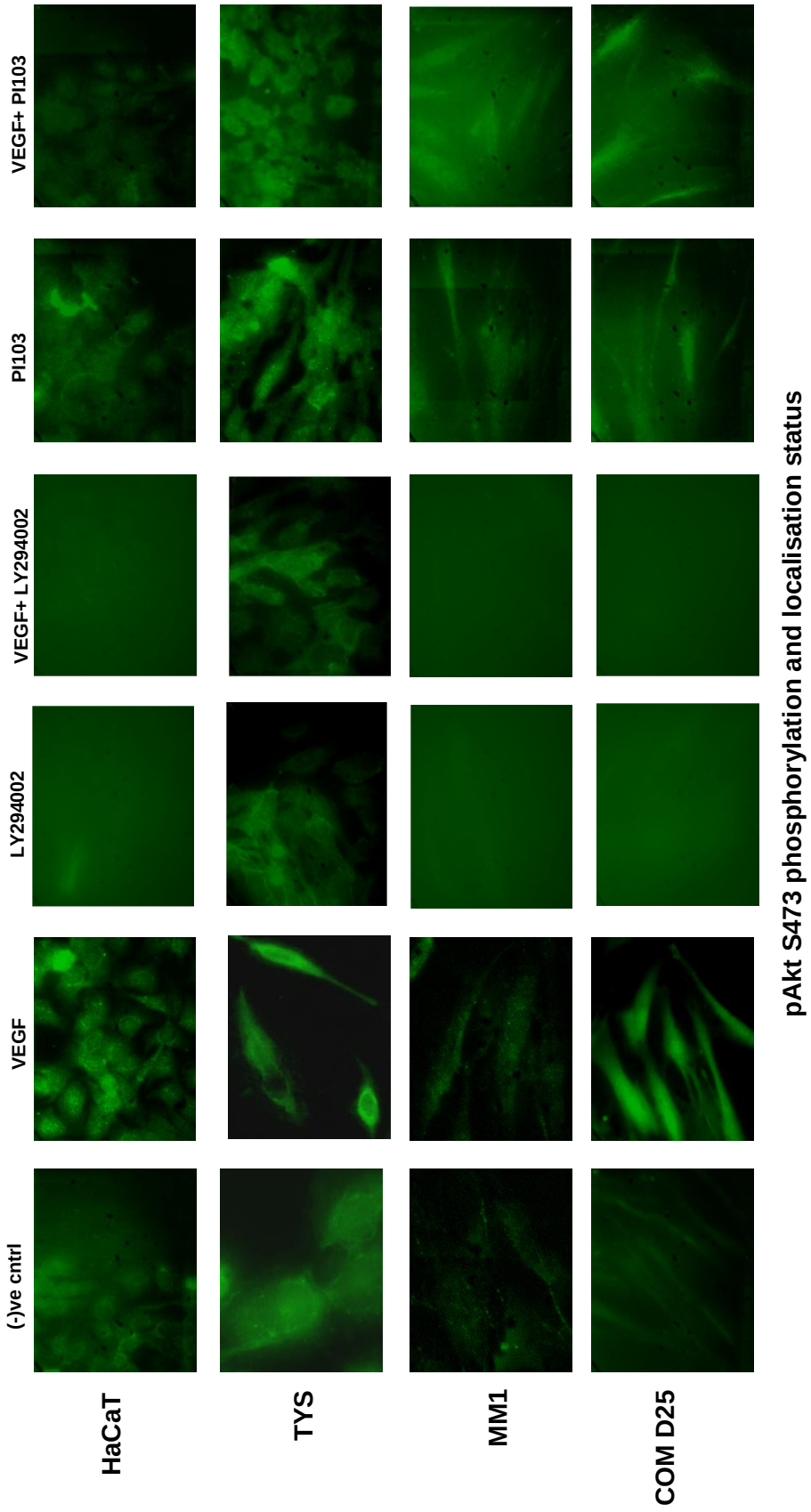


Fig 4.6 (A)

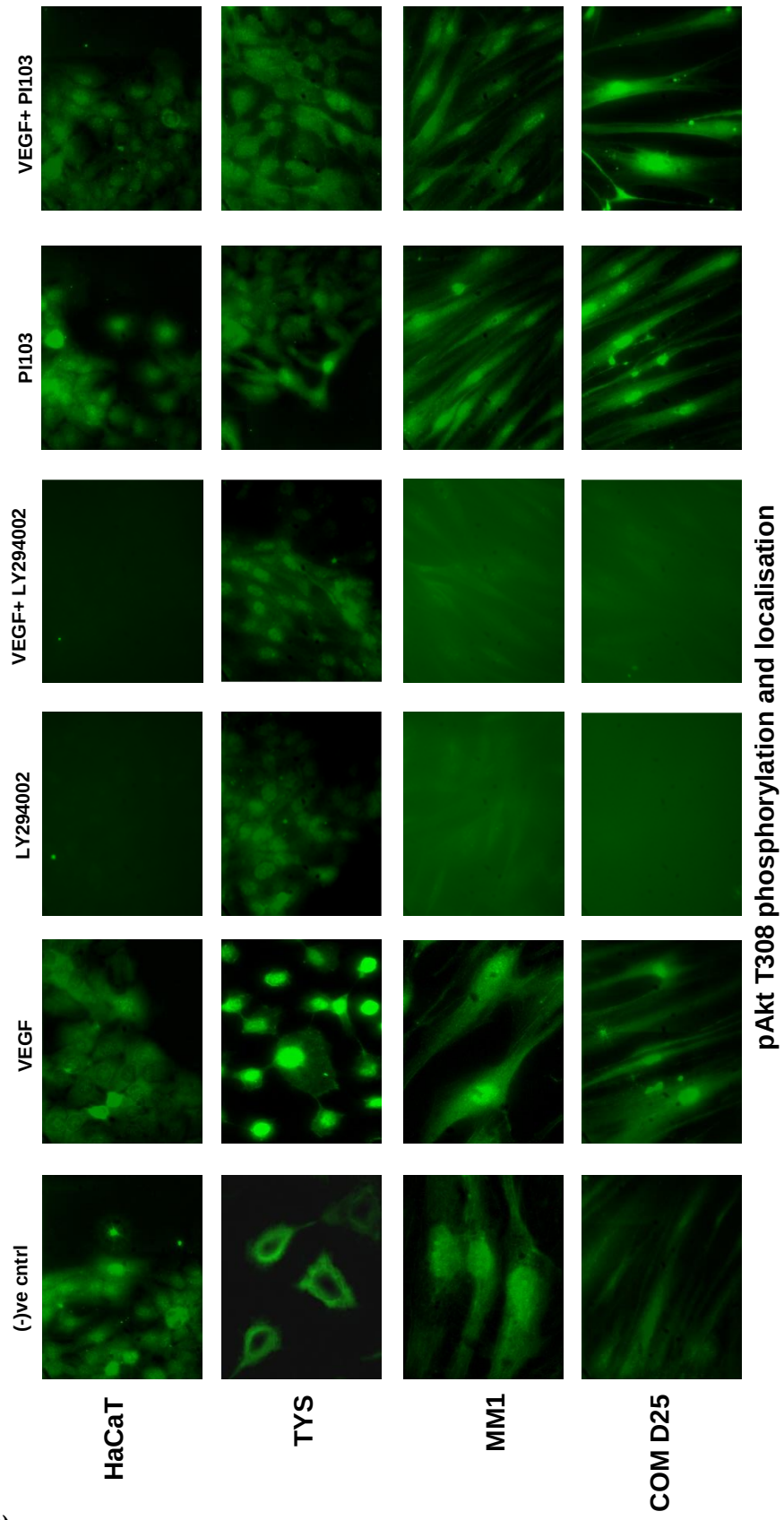

pAkt T308 phosphorylation and localisation
HaCaT
TYS
MM1
COM D25
(-)ve cntrl
VEGF
LY294002
VEGF+ LY294002
PI103
VEGF+ PI103

Fig 4.6 (B)

Figure 4.6 Immunofluorescence assay.
Cells were fixed after 24 hours and immunolocalised for pAkt with a fluorescent labelled antibody. VEGF and serum-induced migration showed strong nuclear localisation of pAkt T308 (B) in contrast to weak cytoplasmic or diffused localisation of pAkt S473 (A). All the fluorescent images were taken at x400 magnification.

4.6 Discussion

We have investigated the relationship between VEGF treatment, Akt activity and cell migration in human cell lines. In summary, we have tested six different cell lines in this study to represent the stages of tumour progression ranging from normal to metastatic cells. Our findings indicate that migration, in response to the addition of exogenous $VEGF_{121,}$ is only upregulated in cell lines originating from dysplastic lesions and tumours. Cancer-associated fibroblasts (CAF) were also stimulated to migrate. In contrast, however, $VEGF_{121}$ did not stimulate normal keratinocytes and normal oral mucosal fibroblast cell migration.

Our migration data is in agreement with previously published studies, where tumour-associated cells such as endothelial cells (Wang *et al.*, 2011) and monocytes (Barleon *et al.*, 1996) were stimulated to migrate in response to VEGF. However, our data for the epithelial cell line, HaCaT, is contrary to data from Yang et al, (2009) who demonstrated that $VEGF_{165}$ enhanced HaCaT cell migration (Yang *et al.*, 2009). Response of cancer cells to VEGF in terms of Akt activation is summarised in Figure 4.7. It should be noted that this is a much simplified view of events. Two cell lines,

TR146 and COM D25, were stimulated to migrate in response to VEGF. These cell lines also displayed an increase in phosphorylation of Akt at S473 and T308 in response to VEGF. The addition of the inhibitor PI103 caused a decrease in the migration of these cell lines.

Tumour progression requires both positive and reciprocal feedback between CAF and cancer cells. Initially, this is manifest by a change in phenotype of normal fibroblasts to that of CAF, which occurs in response to various growth factors, including VEGF, which are secreted by the tumour cells (Cirri and Chiarugi, 2011). However, it may be that these stromal cells themselves are predisposed to respond in a certain way to these growth factors and normal fibroblasts are not (Schor *et al.*, 1991). The CAF act as a source of various types of protease activity (Joyce and Pollard, 2009, Pietras and Ostman, 2010) allowing the cells to play a role in the invasive and metastatic process by remodelling the extracellular matrix. The CAF may also stimulate epithelial to mesenchymal transition (EMT) of tumour cells through secretion of various growth factors or the response to breakdown products of the extracellular matrix (Bhowmick *et al.*, 2004, Pietras and Ostman, 2010). The data presented here is the first evidence that oral cancer-associated fibroblasts are stimulated to migrate by exogenous VEGF treatment.

Experiments using different migration assays and the small molecule PI3K-Akt pathway inhibitors, LY294002, show that treatment of cancer-associated fibroblasts, cells from a dysplastic lesion and adeno squamous carcinoma cells with LY294002

alone, is sufficient to inhibit VEGF-induced migration but not to inhibit oral cancer cells. PI103 appears to be a better inhibitor of oral cancer cell migration and phosphorylation of Akt. This may be due to the fact that PI103 can act at multiple places on the PI3K-Akt pathway. PI103 also blocks mTORC2 from phosphorylating Akt at S473 and mTORC1 further downstream (Dowling *et al.*, 2010). This suggests that PI103 has three chances of blocking activity on one pathway. However, the affects seen here could be PI103 affecting other pathways. In the long term, when considering PI103 as a possibly therapeutic agent, the multi-target effects may be fortuitous and ultimately represent an efficacious strategy for blocking metastases.

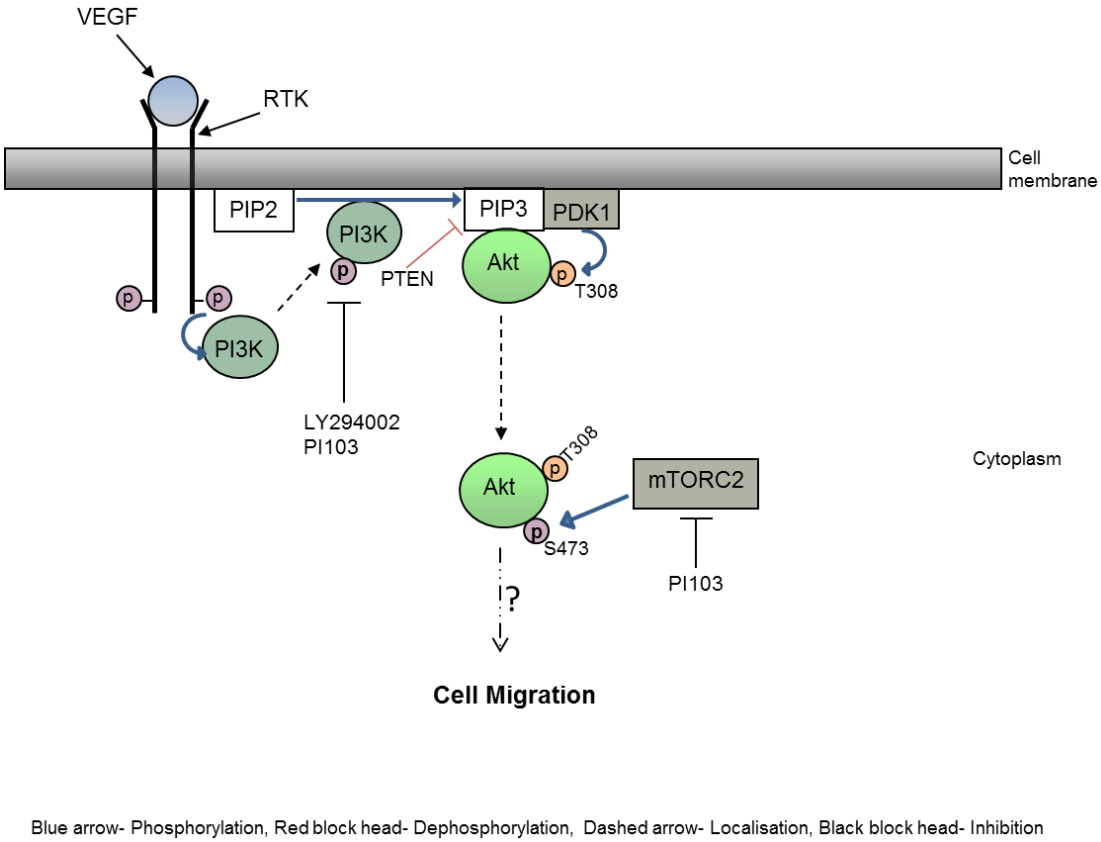


Figure 4.7 Proposed PI3K-Akt signal transduction pathway in oral cancer cell migration.

Akt is phosphorylated at Thr 308 residue by PI3K and at Ser 473 residue by mTORC2. Phosphorylated Akt then stimulates oral cancer cell migration. RTK-Receptor Tyrosine Kinase, PIP2-Phosphoinositide 4,5- biphosphate, PIP3-Phosphoinositide 3,4,5- triphosphate, PI3K- Phosphoinositide 3-kinase, PTEN-Phosphatase and tensin homologue, PDK1-Phosphoinositide-dependent kinase-1, mTORC2- Mammalian target of rapamycin complex-2 (Islam *et al.*, 2014).

Our data support a model where the stimulation of PI3K-Akt activity by VEGF is a mechanism that drives cell migration in CAF and cancerous cells. Akt has been shown to be critically involved in VEGF-induced endothelial cell migration (Morales-Ruiz *et al.*, 2000). Full Akt kinase activity is dependent upon phosphorylation at residues T308 and S473 and this is greatly increased by growth factor receptor signalling (Bozulic and Hemmings, 2009). Regardless of the activation mechanism, once phosphorylated, Akt loses its PIP3 binding requirement and translocates to distinct subcellular compartments, including the nucleus, mitochondria and other organelles (Chin and Toker, 2009). Akt then transduces the signal by phosphorylating numerous substrate proteins, including both cytoplasmic and nuclear proteins. Accordingly, it is not unexpected that Akt activity can be found in both the cytoplasm and nucleus (Rosner *et al.*, 2007). Although it has been suggested that differential phosphorylation of T308 and S473 may modulate the substrate selectivity of Akt, a clear picture of this is yet to emerge (Bozulic and Hemmings, 2009). In this study we have also found nuclear localisation of pAkt T308 in VEGF induced migrated oral adeno-carcinoma cells, whereas pAkt S473 was

mostly diffuse or localised in the cytoplasm. The Ringel group showed that the localisation of activated Akt differs between the two forms of thyroid cancer, but nuclear localisation is associated with tumour invasion in both subtypes (Vasko *et al.*, 2004). Although Akt has been reported to be rich in the nucleus in many cancer cells, the mechanism of translocation, biological importance and activity have not yet been confirmed (Wang and Brattain, 2006).

Chapter 5 . Phosphorylation of Akt in alcohol, tobacco and HPV-induced HNSCC

5.1 Background

HNSCC (Head and Neck Squamous Cell Carcinoma) includes cancers that involve the oral cavity, pharynx and larynx. Each year there are approximately 400 000 cases of cancer of the oral cavity and pharynx, with 160 000 cancers of the larynx, resulting in approximately 300 000 deaths (Boyle and Levin, 2008). It is the sixth most common type of cancer worldwide with a five year survival rate of 40-50%, which has shown only moderate improvement over the last two decades (Al-Sarraf, 2002). Head and neck cancer is strongly associated with lifestyle and certain environmental risk factors such as alcohol and tobacco use, some chemical used in workplaces, UV light and some strains of viruses including HPV (Ridge *et al.*, 2008). Alcohol and tobacco play significant role which have a synergistic effect on HNSCC (up to 100 times higher for both) (Neville and Day, 2002). HPV infection has been detected in around 20% of all cases (Gillison *et al.*, 2013).

Recent studies have focused on the genetic and epigenetic alterations of HNSCC, providing a better understanding of the molecular events underlying the pathogenesis of HNSCC (Mao *et al.*, 2004, Tan *et al.*, 2013). One of the most frequently altered signaling pathways in HNSCC is the PI3K/Akt cascade (Tan *et al.*, 2013). Akt, also known as PKB, is a serine-threonine protein kinase and is central to the phosphatidylinositol 3' kinase (PI3K) signaling pathway (Altomare *et al.*, 1995, Fresno Vara *et al.*, 2004, Bellacosa *et al.*, 2005). PI3K is activated by tyrosine-kinase transmembrane receptors and other signaling intermediates, such as Ras oncogenes

and G proteins (Rodriguez-Viciana *et al.*, 1994). PI3K then phosphorylates PtdIns (4,5) P2 (PIP2) yielding PtdIns (3,4,5) P3 (PIP3), which serves as an anchor for intracellular proteins (primarily mediated by pleckstrin homology domains), including Akt amongst others. Membrane-bound Akt is phosphorylated at T308 in the catalytic domain by the kinase PDK1 and at S473 in the regulatory domain by mTORC2 (Alessi and Cohen, 1998, Sarbassov *et al.*, 2005). Full Akt kinase activity is dependent upon phosphorylation at both T308 and S473 residues and this is greatly increased by growth factor receptor signaling (Bozulic and Hemmings, 2009). PIP3 is converted back into PIP2 through the action of the lipid phosphatase PTEN, thus terminating the PI3K-initiated signal and avoiding further Akt activation (Song *et al.*, 2012). Growth factor receptor over-expression (Sweeny *et al.*, 2012, Thariat *et al.*, 2012), mutation and down-regulation of PTEN protein (Squarize *et al.*, 2013) and amplification of the PIK3CA gene (the gene coding for the catalytic unit of PI3K) (Pedrero *et al.*, 2005) can lead to increased Akt activity. Enhanced Akt activity has indeed been found in 20 to 60% of tumour samples and in the majority of HNSCC-derived cell lines (Pedrero *et al.*, 2005, Mandal *et al.*, 2006, Moral and Paramio, 2008, Amornphimoltham *et al.*, 2011). Once Akt is phosphorylated and activated, it is capable of phosphorylating multiple substrates generating diverse cellular processes, such as metabolism, proliferation, survival and protein synthesis (Lindsley, 2010).

An increasing number of mucosal changes and cellular atypia occur over large areas of the carcinogen-exposed upper aero-digestive tract epithelium, which initiate the

stepwise carcinogenesis process in HNSCC. Acquisition of a transformed phenotype and accumulation of specific molecular genetic events are associated with this process (Slaughter *et al.*, 1953, Liotta *et al.*, 1991, Mao *et al.*, 1996, Grandis *et al.*, 2000, Rosin *et al.*, 2000, Papadimitrakopoulou *et al.*, 2001); yet histopathological evaluation remains the time honoured method in risk assessment of carcinoma lesions. In a search for better biological models of risk, Akt activation was recently identified as an early cellular response to carcinogen exposure and may be a significant step in environmental carcinogenesis (West *et al.*, 2003). Akt activation has also been found to correlate with squamous cell carcinoma progression from normal epithelium to invasive cancer (Amornphimoltham *et al.*, 2004).

5.2 Aims and Hypothesis

The aim of this study was to investigate the role of demographic, pathological and major risk factors (Smoking, alcohol and HPV) on the activation of Akt (phosphorylation of Akt at Threonine 308 and Serine 473) in HNSCC and to determine their prognostic role.

It was hypothesised that lymph node metastasis in HNSCC patients would be associated with phosphorylation of Akt at both residues as they were found to be responsible for head and neck cancer cell migration. It was also hypothesised that Akt at both residues would be found to be highly activated in VEGF positive HNSCC and associated with at least one of the risk factors, if not all.

5.3 Materials

The following materials were used in this study.

Table 22 List of materials

Name	Source	Catalogue number
Orbital shaker	Stuart Scientific, Staffordshire, UK	-
Water bath	Grant Instrument, Cambridge, UK	-
Immuno pen	Dako, Cambridgeshire, UK	S200230-2
Xylene	VWR BDH, PA, USA	28975.325
Ethanol , denatured	VWR BDH, PA, USA	20827.365
Sodium citrate trisodium dihydrate	Sigma-Aldrich, St. Louis, MO, USA	S4641
Hydrogen peroxide	Sigma-Aldrich, St. Louis, MO, USA	H1009
Trizma® Base	Sigma-Aldrich, St. Louis, MO, USA	T1503
Sodium chloride	Sigma-Aldrich, St. Louis, MO, USA	S3014
Tween-20	Sigma-Aldrich, St. Louis, MO, USA	P1379
DAB	Sigma-Aldrich, St. Louis, MO, USA	D5637
Mayer's Haematoxyline	Sigma-Aldrich, St. Louis, MO, USA	S3309
Shandon Bluing agent	Thermo Scientific, Waltham, MA, USA	6769001
DPX mounting media	VWR BDH, PA, USA	360294H
Normal goat serum	Vactor laboratories, Burlingame, CA, USA	S1000
Phospho-Akt (Ser473)(D9E) XP Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	4060
Phospho-Akt (Thr 308) (C31E5E) Rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA	2965
Akt (pan) (C67E7) Rabbit	Cell Signaling Technology Inc., Danvers,	4691

mAb	MA, USA	
SignalStain® Boost IHC detection reagent (HRP, Rabbit)	Cell Signaling Technology Inc., Danvers, MA, USA	8114
Blocking peptide for #4060	Cell Signaling Technology Inc., Danvers, MA, USA	1140
Blocking peptide for #2965	Cell Signaling Technology Inc., Danvers, MA, USA	1145B

5.4 Experimental procedure

5.4.1 Patients

Ethical approval (ID: LEC271/03) was granted for the prospective collection of tissues which were stored at the Tayside Tissue Bank. In total 64 HNSCC and 11 normal oral mucosal tissues (from non-tumour patients) were collected from patients treated at Ninewells Hospital, Tayside. Baseline data obtained from patient charts included age, sex, histology, site, drinking and smoking status, nodal involvement, survival and follow-up data. Patients were followed-up for a total of 66 months (median, 40 months) after diagnosis.

Some continuous variables (such as age, drinking and smoking status) were changed into categorical variables in this study with clear justification (statistical and/or clinical reasons). Age was grouped as '<65 years of age' and '≥65 years' because approximately 50% patients were above/below 65 and a growing number of patients

with Head and Neck Squamous Cell Carcinoma (HNSCC) are age 65 and older (VanderWalde *et al.*, 2013). Patients who consumed alcohol were referred to as 'drinkers' throughout this study. Drinkers are categorised as non-drinkers, medium or moderate drinkers (less than 7 units per week for women and 14 units per week for men or occasional or social, regarded as low risk group by NIAAA), and heavy drinkers (over 7 units per week for women and 14 units per week for men) according to National Institute of Alcohol Abuse and Alcoholism (Niaaa, 2014). Smoking status was also classified as non- or light smokers (less than 5 cigarettes per day) and smokers (more than 5 cigarettes per day) after reviewing the literature (Fagan and Rigotti, 2009, Husten, 2009, Shiffman, 2009).

HNSCC tissues were stained for VEGFA expression by IHC (Islam *et al.*, 2014) and tissues with IHC scores of more than 3 were selected and regarded as positive. Tissues were also analysed for HPV (6, 16 &18) DNA by PCR, automated DNA sequencing and the SPF_{10}-$LiPA_{25}$ method (Sailan, 2010).

5.4.2 Immunohistochemistry

The paraffin-embedded tissues were cut into 5-µm sections, dewaxed in xylene and then rehydrated in serial ethanol solutions, before washing in distilled water for 5 minutes. 58 VEGFA positive HNSCC and 11 normal mucosal samples were then probed with pAkt T308 (#2965) and pAkt S473 (#4060) antibodies according to the manufacturer's instructions (Cell Signaling Technology Inc., Danvers, MA, USA). In brief, after the deparaffinisation and rehydration process, antigens were unmasked

by boiling in 10mM Sodium citrate buffer (pH 6.0) using a microwave, followed by maintenance at a sub-boiling temperature for 10 minutes and then cooling for 30 minutes on the bench top. 3% (v/v) H_2O_2 was then used as a peroxidase blocker and TBST (Tris buffered saline with 0.1% v/v Tween 20) for washing. Sections were then blocked with 5% (v/v) normal goat serum (NGS) plus TBST for 1 hour at room temperature. Sections were then incubated with antibodies against pAkt S473 (1: 50) and pAkt T308 (1:50) diluted in 5% (v/v) NGS/TBST in a humidified chamber overnight at 4°C. After equilibration, sections were then washed three times with TBST and then incubated in signal stain boost detection reagent (HRP, rabbit # 8114, Cell Signaling Technology) for 30 minutes at room temperature. Visualisation was achieved by incubation with 3, 3'-diaminobenzidine (DAB) (Sigma-Aldrich, MO, USA) for 5 minutes and counterstaining with Mayer's haematoxylin (Sigma) and eosin. Rehydration and mounting processes were then followed as described in the instruction manual (Cell Signaling Technology). Normal oral mucosal tissues were used as negative controls. The pAkt S473 and pAkt T308 antibodies were blocked using the respective blocking peptide (#1140 and 1145B, respectively, Cell Signaling Technology) by adding twice the volume of peptide as volume of antibody used, in a total volume of 100 μl. These tissues were also used as negative control. A detail optimisation method is described in Appendix 14.

5.4.3 IHC score

According to the scoring systems that have been reported previously in the literature (Malik *et al.*, 2002, Tang *et al.*, 2006) with some modifications, pAkt staining scoring was performed as follows: stained sections were visualised using a light microscope at high power field and were evaluated by three observers without prior knowledge of the patients' characteristics. An intra-class correlation (inter-observer correlation) analysis using a mixed model and testing for consistency gave a Chronbach's alpha of more than 0.8. The cells showing cytoplasmic and /or nuclear staining were judged as positive. Five high power fields were selected randomly under the microscope. The average percentage of positive staining was calculated for each field. The average percentage of tissue staining was designated as 0 when less than 10% was stained, 1 when 10-25%, 2 when 25-50%, 3 when 50-75% and 4 when >75% of tissues stained. The intensity of tissue staining positively was categorised as follows: 0, no appreciable staining in tissues; 1, barely detectable staining as compared with stromal elements; 2, readily appreciable brown staining distinctly marking cell cytoplasm and/or nucleus; and 3, dark brown staining in tissues completely obscuring cytoplasm and/or nucleus. Scoring was performed according to the product of staining intensity and average percentage of tissue staining positively ranging from 0–12. In the following analysis, the level of Akt phosphorylation was evaluated using the pAkt index either as a continuous variable directly or categorised as no phosphorylation (IHC score 0), low phosphorylation (IHC score 0.1-2.0), medium phosphorylation (IHC score 2.1-5.0), high

phosphorylation (IHC score 5.1-12.0) after reviewing a number of studies (Stal *et al.*, 2003, Kirkegaard *et al.*, 2005, Lim *et al.*, 2005, Messersmith *et al.*, 2005, Ogino *et al.*, 2005, Schmitz *et al.*, 2005, Bose *et al.*, 2006, Tokunaga *et al.*, 2006, Wu *et al.*, 2008, Glynn *et al.*, 2010, Scartozzi *et al.*, 2012).

5.4.4 Statistics

Data were analysed using the statistical package IBM SPSS 19.0. Comparisons between the tissues (HNSCC and normal) regarding the Akt phosphorylation were carried out using a Mann–Whitney U test. Associations between categorical demographic, pathological and behavioural factors were investigated using cross tabulation and Pearson chi-square test. Associations between these variables and continuous immunohistochemical parameters were evaluated with general linear models (both univariate and multivariate). Bonferroni's correction for multiple comparisons was applied where appropriate.

Patients' characteristics and pAkt IHC score were analysed for possible association with overall survival by univariate and multivariate Cox proportional hazard models. Overall survival was defined as the time between diagnosis date and death or last follow-up date. Initially, explanatory factors were screened for univariate associations with death, using a method appropriate to the distribution of the data. If the two-sided P value was <0.300 for any variable it was considered as a candidate in multiple regression models (Hosmer-Lemeshow criterion). Variables with $P \geq 0.300$ were discarded at this stage. The assumption of proportional hazards was checked

for independent variables by plotting the logarithm of the cumulative hazards functions. Starting with the set of variables identified for inclusion from the previous steps, a multiple Cox regression model was built using a step-wise approach. All tests were two-sided, using the 5% significance level. All the statistical results were summarised in Appendix 16.

5.5 Results

5.5.1 Analysis of patient details

58 HNSCC patient details were analysed in this study (patient details can be found in Appendix 13). 33 (57%) were male and 25 (43%) female with ages ranging from 36 to 97 years (median age 64 years). 42 (72%) were smokers and 16 (28%) non-smokers. 7 patients (12%) were non-drinkers, 21 (36%) medium or moderate drinkers and 30 (52%) were heavy drinkers. 31 (53%) patients had negative and 27 (47%) had positive nodal metastasis. Half of the cohort was HPV positive. 43 (74%) patients had T1/T2 and 15 (26%) had T3/T4 tumour size with 5 (9%) of them grade I, 39 (67%) of them grade II and 14 (24%) of them grade III. These data are summarised in Table 23.

Table 23 Demographic, behavioural and pathological data by pAkt status

		Akt T308 phosphorylation status				Akt S473 phosphorylation status			
	n	High (n = 40) %	Medium (n = 8) %	Low (n = 10) %	p	Medium (n = 5) %	Low (n = 30) %	None (n = 23) %	p
Gender									
Male	33	62	63	30	0.109	60	50	65	0.737
Female	25	38	37	70		40	50	35	
Age									
<65 years	30	50	50	60	0.233	80	43	57	0.376
≥65 years	28	50	50	40		20	57	43	
Location									
FOM	10	15	25	20		20	17	17	
RMT	8	10	13	30		0	10	22	0.094
SP	5	10	0	10	0.217	40	10	0	
Tong	27	53	50	20		20	47	53	
Alv	4	5	0	20		0	10	4	
Other	4	7	12	0		20	6	4	
Tumour size									
T1-T2	43	70	25	90	0.173	60	73	78	0.213
T3-T4	15	30	75	10		40	27	22	
Grade									
I	5	8	12	10		0	7	13	
II	39	65	75	70	0.523	80	66	65	0.788
III	14	27	13	20		20	27	22	
Lymph node metastasis									
Positive	27	58	12	30	0.018	40	50	43	0.327
Negative	31	42	88	70		60	50	57	
HPV status									
Positive	29	40	63	80	0.028	40	43	61	0.301
Negative	29	60	37	20		60	57	39	
Smoking									
Yes	42	82	50	50	0.022	80	70	74	0.449
No	16	18	50	50		20	30	26	
Alcohol									
Non-drinker	7	10	0	30	0.027	0	17	9	0.968
Med drinker	21	27	62	40		40	33	39	
Heavy drinker	30	63	38	30		60	50	52	

Note: The general linear model was used for hypothesis testing of the relationship between categorical variables and pAkt. The P value was obtained from univariable analysis. Percentages represent the column percentages within variable so that the balance between the pAkt groups can be assessed.

5.5.2 Immunohistochemistry for Akt phosphorylation

Both normal and VEGF-positive carcinoma patient samples were stained with pAkt S473 and pAkt T308 antibodies. Some samples which were highly stained for pAkt

S473 and pAkt T308 were then selected and tested with the blocking peptide for the respective antibody and were used as negative controls (Figure 5.1A). No staining was observed in the blocking peptide treated tissues and this confirmed the specific binding of the antibodies. Normal tissue samples were also regarded as negative controls and the level of Akt phosphorylation at T308 was higher in the HNSCC group than the control (median 5.8 vs 2.0, $P < 0.001$) (Figure 5.1B). Normal tissue adjacent to the tumours was also tested and the resultant data indicated that there was very low or no pAkt T308/pAkt S473 presents (Figure 5.1C). There is also some evidence to suggest higher levels of pAkt S473 in the cancer group than the controls ($P = 0.054$) (Figure 5.1B). There is a statistically significant difference between pAkt T308 and pAkt S473 levels in the cancer patients (pAkt T308, median 5.8 vs pAkt S473, median 0.3, $P < 0.001$) (Figure 5.1B). VEGFA is not correlated with pAkt T308 ($r = 0.062$, $P = 0.644$) and pAkt S473 ($r = 0.181$, $P = 0.175$). A stepwise progression of cancer including epithelial to mesenchymal transition (EMT) (Figure 5.2A & 5.2B) and migrated cells in stroma (Figure 5.2C) is observed in pAkt T308 stained sections. Table 1 compares the characteristics between pAkt expression groups. All the samples were found to be phosphorylated at Akt T308 so there is no 'no phosphorylation' group for this residue. On the other hand, there is no 'high phosphorylation' group for pAkt S473.

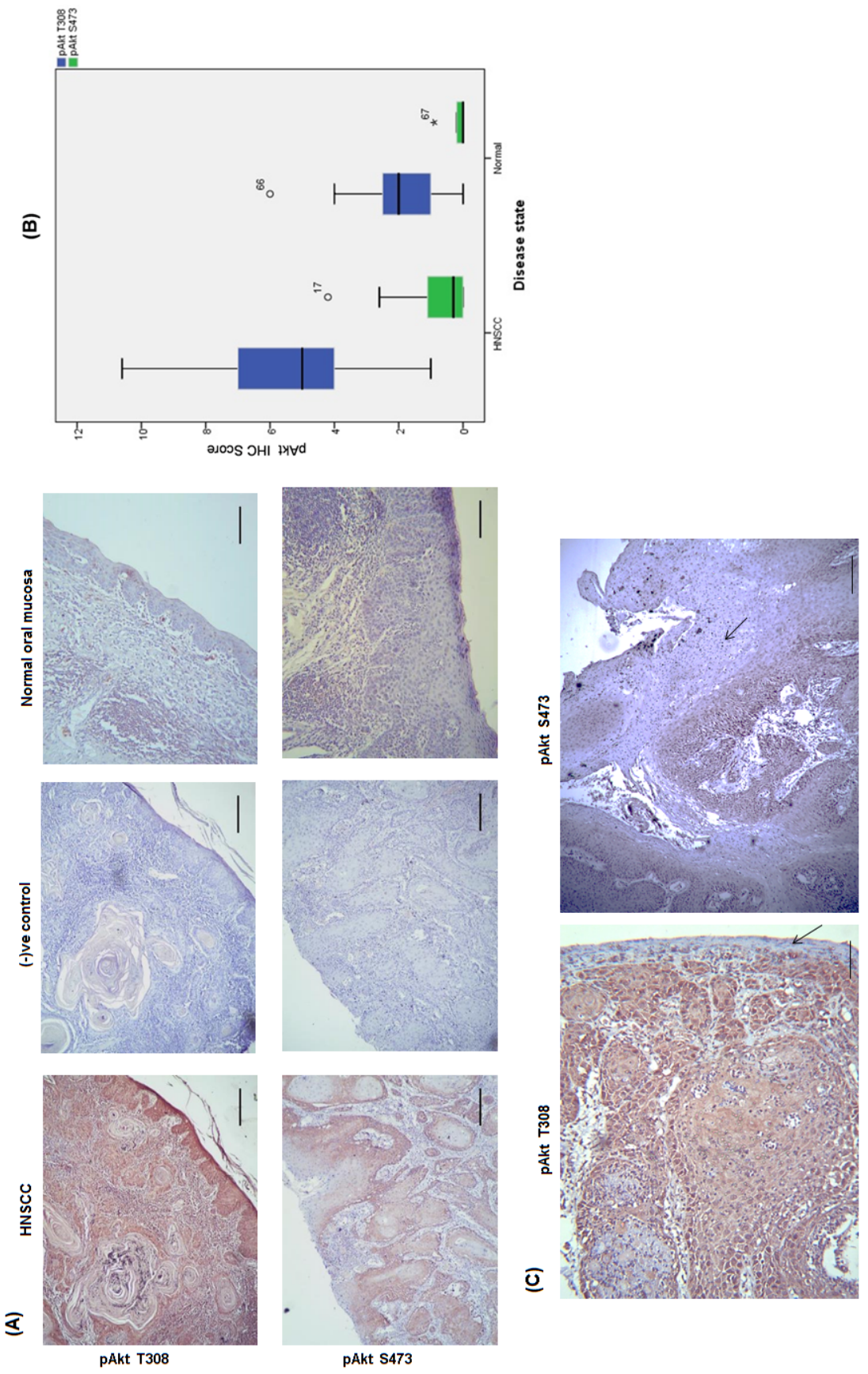


Figure 5.1VEGF positive carcinoma tissues were stained with pAkt antibodies.

Blocking peptides were used to test the efficacy of the antibodies and were tested on duplicate sections of those that had been highly stained for pAkt S473 and pAkt T308 using the antibodies alone. These were used as negative controls. Normal tissues were also used as negative controls, where no staining was observed. All the representative images were taken at x200 magnification except for those stained for pAkt T308, which were taken at x100. **(A)** pAkt S473 and pAkt T308 showed more intense staining in HNSCC tissues compared to normal tissues ($P < 0.05$). **(B)** Phosphorylation of Akt at residue T308 was found to be significantly higher in HNSCC patient samples compared to phosphorylation at S473 ($P < 0.001$). **(C)** Phosphorylation status of Akt in non-tumour part of cancer tissues. Very weak or no phosphorylation of Akt both at T308 and S473 was observed in the non-tumour part of HNSCC tissues. The arrow indicates the non-tumour area of the tissues. Images were captured at x200 magnification. Scale bar= 5mm.

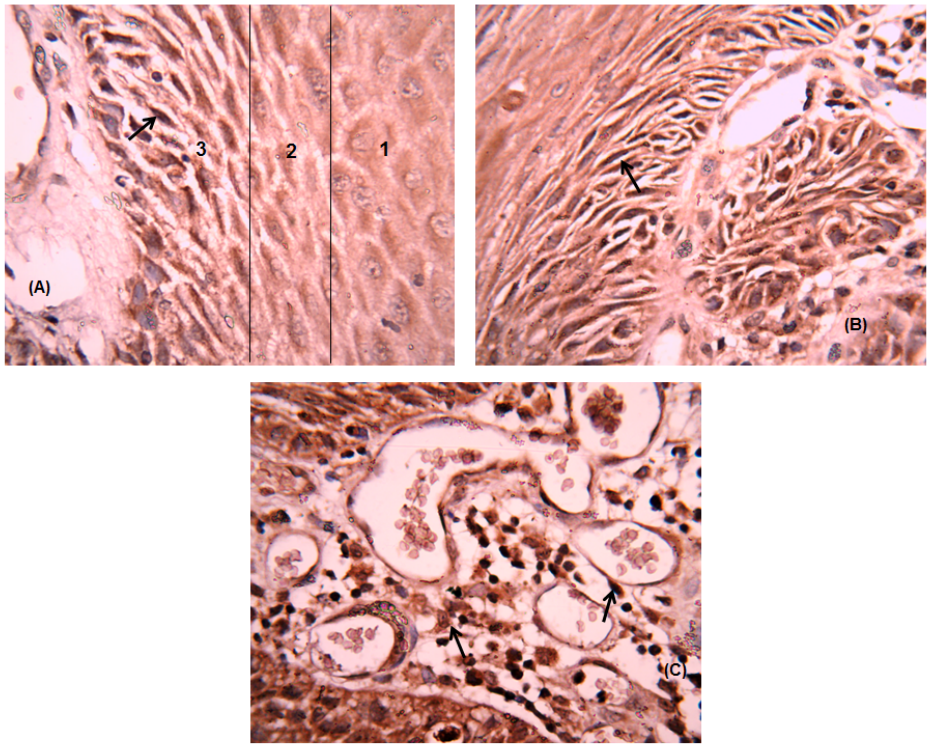


Figure 5.2 EMT in pAkt T308 stained HNSCC.

Akt T308 phosphorylated cancer cells that had undergone epithelial to mesenchymal transition. (A) Attached cancer cells were detached by breaking cell-cell junctions and changed their shape from epithelial to mesenchymal like cells (EMT) before migrating to the stroma. 1, 2 and 3 showed the stepwise transition whereas 1 represents cells with tight junction, 2 represents cells with loose junctions and 3 represents mesenchymal type cells. (B) EMT of cancer cells before migration. Arrows in (A) and (B) represents mesenchymal type cancer cells that had undergone EMT. (C) Migrated cancer cells in stroma (arrows). All the representative images were taken at ×400 magnification.

5.5.3 Association of patient characteristics with Akt phosphorylation

Smoking and alcohol were found to be independent risk factors for phosphorylation of Akt at T308 (P = 0.022 and 0.027, respectively) but not for pAkt S473 (P = 0.449 and 0.968, respectively). HNSCC with nodal metastasis was associated with a higher level of pAkt T308 than HNSCC without nodal metastasis (P = 0.018). Smokers and HNSCC with nodal metastasis were found to have higher levels of Akt phosphorylated at T308 than non-smokers (P = 0.022) and HNSCC without modal metastasis (P = 0.018) patients, respectively. HPV-negative patients exhibited higher levels of pAkt T308 compared to those who were HPV positive (P = 0.028). There is some evidence to suggest that heavy drinking patients had higher levels of pAkt T308 than medium drinkers (P = 0.063).

Adjusted by four independent variables, smoking, drinking, nodal status and age, the general linear model accounts for 65.2% ($R^2 = 0.652$) of the total variation in pAkt T308 levels (Table 24). This model's significance statistics for the F-statistic (P < 0.001)

indicate that there is only a very small chance that the observed correlation between one or more of the independent variables and the dependent variable is due solely to random sampling error.

Smoking ($P = 0.027$), drinking habit ($P < 0.001$) and age ($P = 0.031$) showed a significant effect on the phosphorylation of Akt at T308. Alcohol and age, alcohol and nodal involvement, smoking and nodal involvement and smoking and alcohol are strongly correlated with the phosphorylation of Akt at T308 in pairwise combination ($P = 0.009$, 0.038, 0.049 and 0.052, respectively). Moreover, smoking, nodal involvement, age and alcohol, nodal involvement, age also have a strong correlation with the phosphorylation of Akt T308 ($P = 0.019$ and 0.022, respectively). None of these independent variables correlated with the phosphorylation of Akt at S473 (univariate and multivariate, $P > 0.05$).

Non-drinking patients had lower levels of Akt phosphorylated at T308 and were more likely to be infected by HPV than heavy drinkers (χ^2 $P = 0.044$ and 0.007, respectively). Also, HPV infected patients were shown to have lower levels of Akt phosphorylated at T308 than the non-infected patients (χ^2 $P = 0.028$).

Table 24 General Linear Model (Multivariate analysis)

Independent variables	**F**	**P**
Corrected Model	4.06	<0.001
Smoking	5.30	0.027
Alcohol	10.56	<0.001
Nodal Status	1.31	0.260
Age	4.98	0.031
Smoking * Alcohol	3.20	0.052
Smoking * Nodal status	4.12	0.049
Smoking * Age	0.01	0.919
Alcohol * Nodal status	3.56	0.038
Alcohol * Age	5.31	0.009
Nodal status * Age	2.80	0.102
Smoking * Alcohol * Nodal status	0.17	0.682
Smoking * Alcohol * Age	0.08	0.774
Smoking * Nodal status * Age	6.00	0.019
Alcohol * Nodal status * Age	5.73	0.022
$R^2 = 0.652$		

Note: After adjusting Smoking, Alcohol, Nodal Status and Age, this model accounts for 65.2% of the total variations in pAkt T308 level. R^2 = Coefficient of determination, F = F-statistics. Dependent variable: pAkt T308 score.

5.5.4 Survival Analysis

Levels of pAkt S473 and pAkt T308, as determined by IHC, were examined for association with overall survival using Cox's proportional hazard model (Table 25). In multivariate analysis, pAkt S473 level, tumour size, alcohol consumption, age and patients' HPV status had significant effects on overall survival (P = 0.005, 0.005, 0.021, 0.007 and 0.004, respectively). The rate of deaths in this small cohorts of HNSCC patients with medium levels of phosphorylated Akt S473 was higher than in those with none and in those with low levels (P = 0.001 and P = 0.036, respectively, Table 3). However, this is an observation of a marker and cannot be attributed to the case of why and how they died. Patients with tumours of size T3/T4 died more rapidly than patients with tumours T1/T2 (HR = 15.2, P = 0.005). Death rates in heavy drinkers were higher than those who consumed no alcohol (P = 0.006). Older patients (65 years of age or over) were more likely to die than those of 65 years of age (P = 0.007). HPV positive patients were more likely to die in this small cohort than those with a negative status (P = 0.004). A number of recent studies however, revealed that the prognosis for HPV-positive patients (especially in oropharyngeal cancer) is better than that for patients with HPV negative tumour independent of age, gender, stage, nodal status and tumour differentiation (Weinberger *et al.*, 2006, Lindquist *et al.*, 2007, Fakhry *et al.*, 2008).

Table 25 COX proportional hazard model- time to death

		Unadjusted HR			Adjusted HR		
		HR	95% CI	P	HR	95% CI	P
pAkt S473	Overall			0.168			0.005
	Med : No	6.27	0.87, 45.3	0.069	438	10.9, 1755	0.001
	Med : Low	1.83	0.38, 8.88	0.456	21.5	1.23, 376	0.036
Tumour size	T3/T4 : T1/T2	4.59	1.39, 15.2	0.013	15.2	2.28, 102	0.005
Alcohol	Overall			0.005			0.021
	Heavy : Non-drinker	15.4	2.74, 86.7	0.002	49.4	3.04, 801	0.006
	Heavy : Moderate	3.19	0.62, 16.5	0.166	2.69	0.35, 20.8	0.343
Age	≥65 : <65	5.41	1.17, 25.05	0.031	46.8	2.81, 781	0.007
HPV	+ve : −ve				89.4	4.05, 1973	0.004
Gender	F : M	1.40	0.42, 4.63	0.581			
Tumour size	Overall			0.310			
Nodal status	+ve : −ve	1.49	0.43, 5.10	0.529			
Smoking	No : Yes	1.45	0.42, 4.95	0.556			
pAkt T308	Overall			0.984			
	Low : High	1.02	0.21, 4.92	0.985			
	Med : High	1.16	0.24, 5.63	0.858			

Note: Unadjusted HR obtained from univariable analysis and adjusted HR from multivariable analysis after adjusting tumour size, alcohol, age, HPV and pAkt S473. All the variables are categorical and HR = exp (B). Abbreviations- HR, Hazard ratio, 95% CI, 95% confidence interval.

5.6 Discussion

To our knowledge this is the first report to show the relationships between the major risk factors for HNSCC (alcohol, smoking and HPV) and Akt activation (both at residues T308 and S473) at the protein level using an immunohistochemical staining method (IHC), in surgically resected specimens. Most studies to date have used IHC

to assess the prognostic value of Akt activation in HNSCC, but have focused only on phosphorylation of residue S473. As the differential phosphorylation of Akt at the two sites may modulate downstream substrate selectivity and subsequent bioactivity (Bozulic and Hemmings, 2009), it is not surprising that Akt phosphorylated at any single site could perform certain cellular bioactivities. Two different mechanisms are involved in phosphorylating Akt, therefore overexpression or amplification of any components in these mechanisms may result in over-phosphorylation at any one of the two sites. It is therefore worth studying the phosphorylation status of Akt at both sites in HNSCC specimens to elucidate their different roles.

As only VEGFA positive HNSCC biopsy samples were selected in this study, no statistically significant correlation was found between VEGF and pAkt. The present study showed that Akt was significantly phosphorylated at T308 in VEGF positive HNSCC rather than S473. Alcohol and smoking were positively correlated with pAkt T308 activation but not with pAkt S473. Moreover, Akt activated at T308 showed a significant relationship with lymph node metastasis, which suggests that pAkt T308 may be concerned with invasion and metastasis. This data is similar to our in vitro data concerning the migration of tumor cells in response to VEGF, which suggests that migration of oral adeno-squamous cancer cells is dependent on Akt T308 phosphorylation. Our study also disclosed that Akt phosphorylated at both residues controls oral cancer cell motility (Islam *et al.*, 2014), but it should be remembered that studies performed with cultured cells or tissue models may

produce different results. *In vivo*, tumor progression requires both positive and reciprocal feedback between the components of the tissue microenvironment and cancer cells (Cirri and Chiarugi, 2011).

The activation of Akt in response to alcohol exposure is an important contributor to the molecular effects of excessive alcohol consumption (Neasta *et al.*, 2011). In 2003, West showed that redundant Akt activation by nicotine and nicotine-derived nitrosamine ketone (NNK) could contribute to tobacco-related carcinogenesis (West *et al.*, 2003). A study by the Gonzalez group in 2005 revealed that Akt activation was correlated with concomitant PI3K accumulation and PTEN down-regulation in HNSCC, reflecting an early biochemical effect in response to nicotine (Pedrero *et al.*, 2005). Combined with these data, our study supports the basic hypothesis that Akt activation (especially at T308) is a key step in the progression of HNSCC caused by alcohol and smoking. HPV infection, another risk factor for HNSCC, was found to be negatively correlated with Akt activation at T308, as HPV positive HNSCC patients showed lower levels of pAkt T308. Non-drinking patients had lower levels of activated Akt at T308 too and there were more HPV positive patients among non-drinkers than amongst the heavy drinkers. Earlier epidemiological research supports this data, that is non-smokers and light or non-drinkers are more likely to have tumours positive for HPV than are heavy smokers and drinkers (Lindel *et al.*, 2001). Molinolo et al. (2012) showed in their study that HPV positive HNSCC patients over-activate Akt at S473 and mTOR (Molinolo *et al.*, 2012). Although we have not

found any association between HPV infection and pAkt S473 activation, this may suggest that there are two different mechanisms of cancer progression initiated by alcohol, smoking and HPV. The study by Kelsey group (2007) strongly supports the emerging view that the aetiology of HPV related HNSCC is distinct from that of HNSCC tumours associated with smoking and drinking (Applebaum *et al.*, 2007). Increased Akt activation at T308 by excessive alcohol and smoking may be responsible for cancer development and progression, including metastasis, whereas HNSCC by HPV infection may over-activate Akt at S473 and be responsible for poor survival.

In this study we show that increased pAkt S473 levels in HNSCC are a strong predictor for poor patient outcome. In the multivariate Cox proportional hazard model, adjusted for well recognised prognostic indicators (e.g. tumor size and age), pAkt S473 status remained a strong predictor. This is corroborated by three other studies which have shown that pAkt activated at S473 is associated with poor prognosis in oral cancer (Lim *et al.*, 2005, Massarelli *et al.*, 2005, Yu *et al.*, 2007). Although further molecular analysis is needed to investigate the mechanism of smoking and alcohol related HNSCC development, we can propose pAkt T308 as a reliable biomarker for smoking and alcohol induced HNSCC progression. A large cohort with a longer follow-up of pre-neoplastic and HNSCC lesions is needed to more accurately define the role of Akt activation in carcinogenesis and to integrate this data into a risk model for carcinoma development and progression.

Chapter 6 General discussion, conclusion and further investigations

6.1 General discussion

EGFR is overexpressed in 90% of HNSCC and correlated with poor prognosis (Pablo Uribe and Sergio Gonzalez, 2011). Cetuximab which inhibits EGFR is the first molecular targeted agent approved for the treatment of both locally advanced and recurrent/metastatic HNSCC (Specenier and Vermorken, 2013, Schmitz *et al.*, 2014). However, only 13% of patients respond to cetuximab (single agent), despite its great clinical promise (Cohen, 2014). New studies have revealed that aberration of Ras/MAPK/ERK and PI3K/Akt pathway is the central mediator of cetuximab resistance in HNSCC (Rebucci *et al.*, 2011, Rampias *et al.*, 2014). Activation of the PI3K/Akt signalling pathway is also found to be responsible for radio-resistance in HNSCC (Saki *et al.*, 2013). Recent studies suggest that the dynamics of a variable clonal sub-population due to tumour heterogeneity may also modify the cellular response to cetuximab (Greaves and Maley, 2012, Kreso *et al.*, 2013). One of the interesting developments in the recent cancer research, is the discovery that tumour cells exhibit both autocrine and paracrine VEGF signalling (Goel and Mercurio, 2013) and that this signalling participates in vital aspects of cancer progression, especially in the function of cancer stem cells and angiogenesis (Beck *et al.*, 2011, Perrot-Applanat and Di Benedetto, 2012, Mittal *et al.*, 2014) . In addition, VEGF can affect the function of fibroblasts and immune cells in the tumour stroma and consequently,

it can affect the host response to tumours (Pietras and Ostman, 2010, Junttila and de Sauvage, 2013).

Studies in this project into the role of Akt in VEGF-induced cancer cell migration have produced potentially conflicting results, which reveal both positive and negative effects of the inhibitors. For example, a specific PI3K inhibitor, LY294002 inhibited the migration of mouth cancer associated fibroblast and oral-adeno squamous cell carcinoma but was unable to inhibit the oral squamous cell carcinoma migration. This paradox could be, in part, explained by different cell types and experimental design i.e. the migration assay format (2D and 3D, concentration gradient and non-gradient environment) and treatment period employed (4 hours, 24 hours and 4 days). LY294002 did not inhibit oral-adeno carcinoma cell migration in the 2D non-gradient format (scratch assay, 24 hours) but effectively inhibited the migration in 2D gradient format (Boyden chamber, 4 hours). PI103 (PI3K and mTOR inhibitor), on the other hand, effectively inhibited the migration of oral cancer cells both in 3D non-gradient (collagen gel assay, 4 days) and 2D gradient (live cell chemotaxis, 24 hours) format, but was unable to inhibit the migration of oral adeno-carcinoma cell in 2D non-gradient format (scratch assay, 24 hours). In addition, intra-tumoural variation reflective of the existence of multiple sub-clonal tumour populations may also contribute. This variation might correspond to an essential, yet unrecognised, determinant for the appearance of secondary drug resistance (Sinha *et al.*, 2013). In general, the data obtained from the *in-vitro* migration assays have revealed that Akt needs to be phosphorylated at both the Thr308 and Ser473 residue

for oral cancer cells to be migrated by VEGF and this stimulation of migration can be blocked by PI103. Thus, VEGF-induced oral cancer cell migration is PI3K and mTROC2 dependent as they are responsible for phosphorylation of Akt at Thr 308 and Ser 473 residues, respectively.

Results obtained from the experiments using cell and tissue systems have also produced conflicting results. Cell migration assays revealed that Akt phosphorylated at both Thr308 and Ser473 residues is responsible for oral cancer cell migration, whereas analysis of HNSCC tissue samples revealed that Akt phosphorylated at Thr308 only is positively correlated with lymph node metastasis. The over-activation of Akt Ser473 only is responsible for patient's worse survival. This contradiction can be explained by the role of the tumour microenvironment (TME). Interaction between cancer cells and the associated components of tumour microenvironment characterise a powerful connection that stimulates cancer initiation, progression and patient prognosis (Joyce and Pollard, 2009). TME continually changes over the course of cancer progression in response to oncogenic signals such as VEGF overexpression by growing tumours and evolving environmental conditions. The influences of TME on metastasis is thus considered as a dynamic process (Quail and Joyce, 2013).

Inconsistent responses to targeted therapies illustrate the requisite for personalised cancer treatment, where the importance of recognising and appreciating the specific intricacy and variability of a tumour is paramount. The data presented exemplify

that to enable the design of efficacious chemotherapeutic regimes, there is an absolute requirement for individual tumours to be precisely characterised.

6.2 Conclusion

In conclusion, the following list summarises the main findings of this project:

1. Phosphorylation of Akt is VEGF concentration, treatment period and cell type dependent.
2. VEGF-induced phosphorylation of Akt and subsequent motility behaviour is cell type and incubation time dependent.
3. VEGF-induced *in vitro* cell migration is also dependent on assay format.
4. Akt needs to be phosphorylated at both the Thr308 and Ser473 residue for oral cancer cells to be effectively stimulated to migrate by VEGF.
5. pAkt Thr308 can be used as a potential biomarker for smoking and alcohol-induced HNSCC and lymph node metastasis.
6. The predictive role of Akt activation in HNSCC and cell migration studies suggests that targeting PI3K/Akt and mTORC2/Akt pathway might be a useful strategy for therapy in this disease. So, PI103 can be used as a potential therapeutic inhibitor in metastatic HNSCC.

In conclusion, our findings suggest that targeting Akt activation might be of interest as part of a combination therapy in HNSCC.

6.3 Further investigations

1. To elucidate the downstream signalling pathway of Akt responsible for oral cancer cell migration.

2. To identify the cellular mechanism of VEGF-induced oral cancer cell migration e.g., EMT or actin re-organisation and understand the associated molecular pathway.
3. To recognise the possible substrates of Akt in the migration of other cell types.
4. To use GFP-pAkt transfected cells in the live cell chemotaxis assay to clarify the localisation of phosphorylated Akt during cell migration.
5. To use 3D organotypic assay to illustrate the possible role of tumour microenvironment on the migration and invasion of oral cancer cells.
6. To apply *in vivo* migration and invasion assays to analyse the invasive cells from the primary tumour and to better understand the cell types and signalling events involved in the tumour microenvironment.
7. To use PI103 in a mouse model of oral cancer to elucidate its therapeutic benefit.
8. To study the role and mechanism of CSCs and the tumour microenvironments on head and neck cancer drug resistance.
9. A large cohort with a longer follow-up of pre-neoplastic and HNSCC lesions is needed to more accurately define the role of Akt activation in carcinogenesis and to integrate this data into a risk model for carcinoma development and progression.
10. Establishing new ways of destroying head and neck cancer cells using nano-particles, or modification of viruses e.g. herpes simplex.

Chapter 7 References

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Chapter 8 Appendices

Appendix 1. Justification and validation of using cell lines

Justification:

Six cell lines of different stages (Normal-dysplastic-cancer) were selected in this project to investigate the phosphorylation status of Akt with their subsequent migration behaviour and compare with each other. We aimed to select all the cell lines (epithelial and fibroblasts) from Head and Neck origin. Justification of using individual cell line is summarised below:

Cell type

HaCaT: Normal skin keratinocyte (Squamous epithelial origin). First reason of selecting HaCaT cell is unavailability of oral mucosal keratinocytes in the laboratory. Although derived from skin squamous epithelial region their morphology and function is similar to oral mucosal squamous epithelial cells.

TR 146: HNSCC (epithelial cells from buccal mucosa)

TYS: Oral adenoid squamous cell carcinoma (An epithelial neoplastic cell line was derived from well-differentiated squamous cell carcinoma that arose in human oral mucosa. The cell line then treated with Sodium-butyrate and transplanted in nude mice. The resulting mass was then histopathologically tested and interpreted as human adenoid squamous cell carcinoma derived from minor salivary gland present in oral mucosa (Yanagawa T. *et ai,*1986). We selected this cell line to compare its pAkt status and migratory behaviour with that of squamous cell carcinoma.

MM1: Normal oral mucosal fibroblast (fibroblast origin)

COM D25: Mouth cancer-associated fibroblast cell (fibroblast origin)

PM1: Dysplastic cell (derived from forehead skin, squamous epithelial origin as described in the literature). The unavailability of dysplastic cell line from oral mucosal origin made us select this cell line. Also, their characteristics were similar to oral mucosal squamous l dysplastic cells.

Validation of cells used:

Cells were tested with epithelial and fibroblast marker to prove their origin.

Experiment type: Immunofluorescence assay

Primary antibodies:

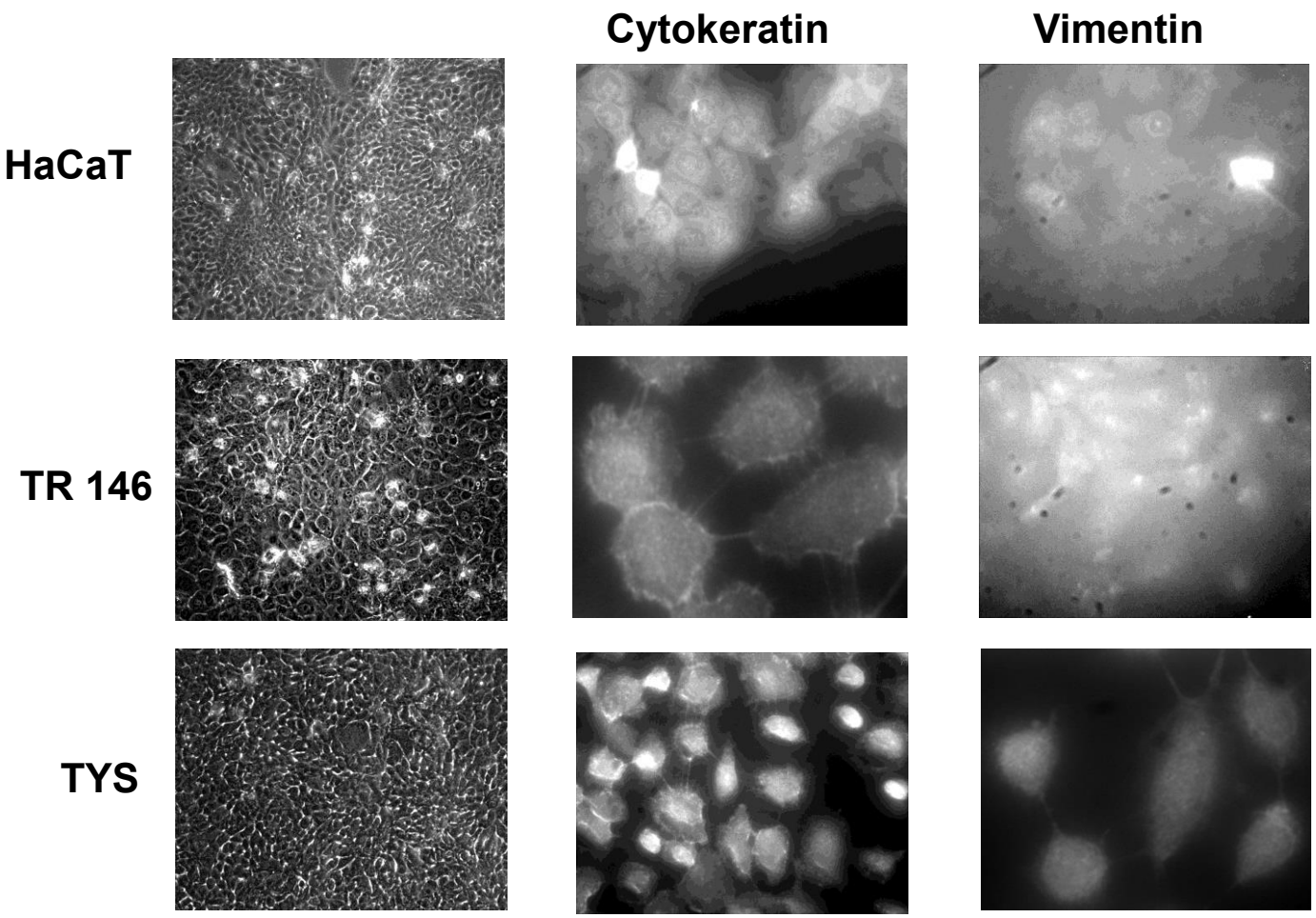
1. Rabbit anti-wide spectrum cytokeratin antibodies (Ab9377, Abcam, Cambridge, AM, USA, Dilution- 1:100)- Epithelial marker
2. Vimentin (D21H3) XP rabbit mAb (#5741. Cell signalling technology, MA, USA. Dilution- 1:100)- Fibroblast marker

Secondary antibody: Goat Anti Rabbit Alexa Fluor 488 conjugated (#4412, Cell signalling technology, Dilution- 1:1000)

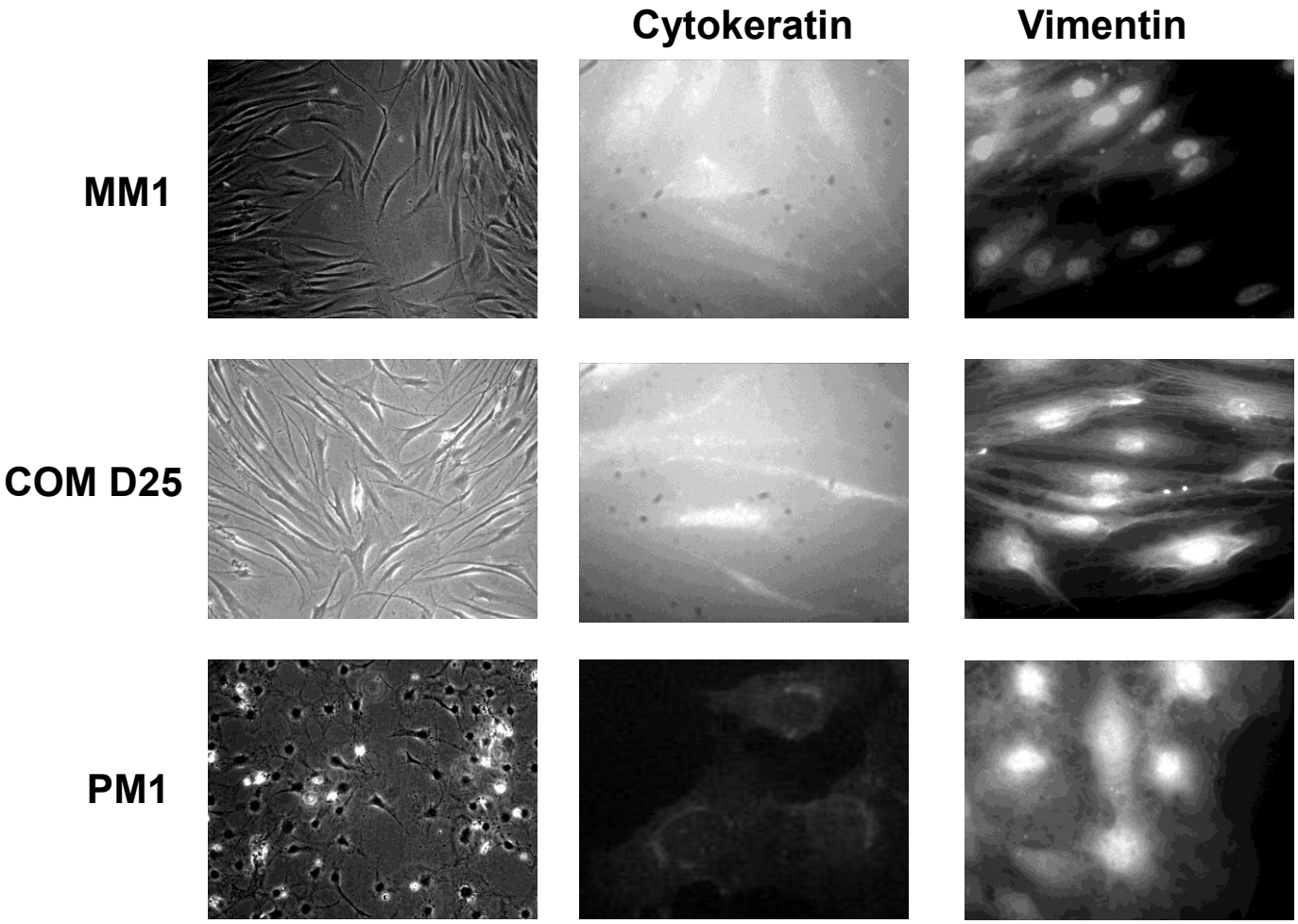
Methodology: All the cells were cultured in serum-free medium.

Immunofluorescence methods as described in Chapter 4.

Results:



HaCaT, TR 146 and TYS showed higher cytokeratin and very low or no vimentin expression that proves their epithelial origin.



MM1 and COM D25 cells showed higher vimentin and very low or no cytokeratin expression that proves their fibroblast origin. PM1 (dysplastic cell) showed high vimentin and very low cytokeratin expression which opposed their origin as described in the literature (Proby *et al,* 2000). This is the reason why this cell line was described in this project as 'cells from dysplastic lesion'.

Appendix 2. Representative WB report (TR146, PM1, VEGF, pAkt S473, pAkt T308)

DATE: 07.06.12	Reducing 'Any kD' SDS PAGE BioRad Pre-cast Gels

EXPERIMENTS

Analysis of cell pAkt (Thr 308) and pAkt Ser 473. Cells were grown up (~confluent PM1 and TR146 80%) in 60mm dishes and then transferred to serum free (SF) medium overnight, prior to the experiment. Cells were then transferred to the test conditions (i.e. SF, or SF + VEGF) for 15 minutes. Cells were then washed with ice-cold PBS and lysed in RIPA buffer containing protease inhibitor.

SAMPLE PREPARATION FOR GELS

Frozen lysates were used for blot. Prior to use, the lysates were defrosted and spun at 13,000rpm for 5 minutes. Samples were then mixed with an equal volume of Laemmli sample loading buffer (BioRAD) including 5% (v/v 2-mercaptoethanol). Samples were heated at 95°C for 5 minutes.

WESTERN BLOTTING

As described in method, onto nitrocellulose using transfer buffer. 15V 42 minutes.

PRIMARY ANTIBODIES

1. anti pAkt Thr 308 # 2965 (Cell Signaling Technology Inc.). Diluted 1:1000 in TBST + 1% (w/v) dried milk (N.B. 5% w/v BSA was recommended by the manufacturer)

2. Anti-pAkt Ser 473 # 4060 (cell Signaling) .Diluted 1:2000 in TBST + 1% (w/v) dried milk.

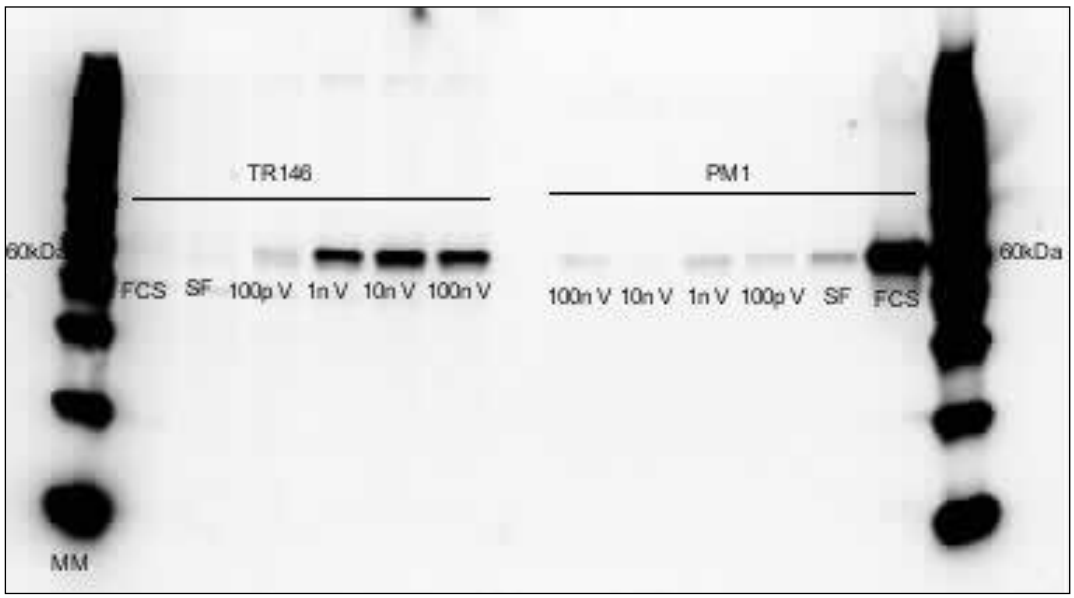
SECONDARY ANTIBODY

1. Goat anti-rabbit HRP# 7074L (Cell Signaling) was used for at a 1:2,000 diluted in 1% (w/v) milk TBST .Washed with TBST .

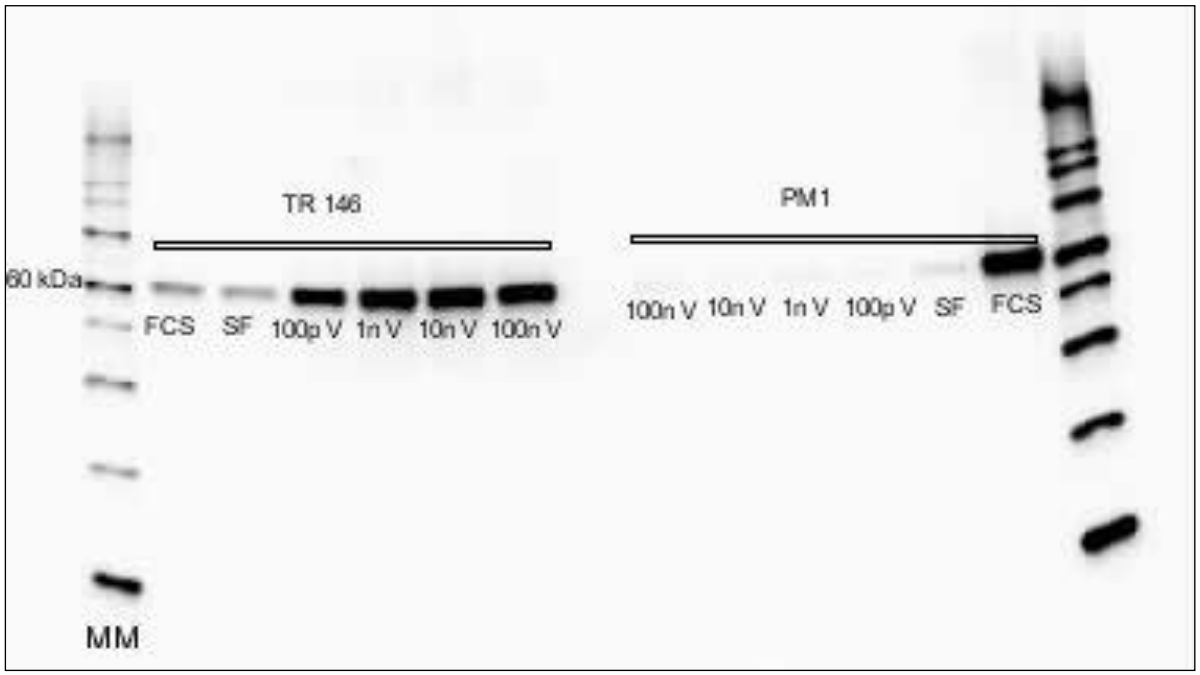
GEL 1 LOADING			
Lane		µl	
1	MM	2.5	Western Blot Anti pAkt Thr 308 1:1000 Biochem lab protocol
2	Positive control (FCS)	15	
3	PM1 SF	15	
4	PM1 SF + 100pg/ml VEGF	15	
5	PM1 SF + 1ng/ml VEGF	15	
6	PM1 SF + 10ng/ml VEGF	15	
7	PM1 SF + 100ng/ml VEGF	15	
8			
9	TR146 SF + 100ng/ml VEGF	15	
10	TR146 SF + 10ng/ml VEGF	15	
11	TR146 SF + 1ng/ml VEGF	15	
12	TR146 SF + 100pg/ml VEGF	15	
13	TR146 SF	15	
14	Positive control (FCS)	15	
15	MM	2.5	

GEL 2 LOADING			
Lane		µl	
1	MM	2.5	Western Blot Anti pAkt Ser 473 1:2000 Biochem lab protocol
2	Positive control (FCS)	15	
3	PM1 SF	15	
4	PM1 SF + 100pg/ml VEGF	15	
5	PM1 SF + 1ng/ml VEGF	15	
6	PM1 SF + 10ng/ml VEGF	15	
7	PM1 SF + 100ng/ml VEGF	15	
8			
9	TR146 SF + 100ng/ml VEGF	15	
10	TR146 SF + 10ng/ml VEGF	15	
11	TR146 SF + 1ng/ml VEGF	15	
12	TR146 SF + 100pg/ml VEGF	15	
13	TR146 SF	15	
14	Positive control (FCS)	15	
15	MM	2.5	

Gel1: pAkt T308



Gel 2: pAkt S473



Appendix 3. Representative WB experiment report (TR 146 VEGF time course pAkt T308)

DATE: 10.10.13	Reducing 7.5% SDS PAGE BioRad Pre-cast Gels

EXPERIMENTS

Analysis of cell pAkt Thr308 and pAkt S473. Cells were grown up (~confluent TR146 80%) in 60mm dishes and then transferred to serum free (SF) medium overnight, prior to the experiment. Cells were then transferred to the test conditions (i.e. SF, VEGF) for 15 minutes,5 hrs and 24 hrs. Cells were then washed with ice-cold PBS and lysed in RIPA buffer containing protease inhibitor.

SAMPLE PREPARATION FOR GELS

Cells were frozen at -20ºC. Prior to use, the lysates were thawed and then spun at 13,000rpm for 5 minutes. Samples were then mixed with an equal volume of Laemmli sample loading buffer (BioRAD) including 5% (v/v 2-mercaptoethanol). Samples were heated at 95°C for 5 minutes.

WESTERN BLOTTING

As described in method, onto nitrocellulose using transfer buffer. 15V 42 minutes.

PRIMARY ANTIBODIES

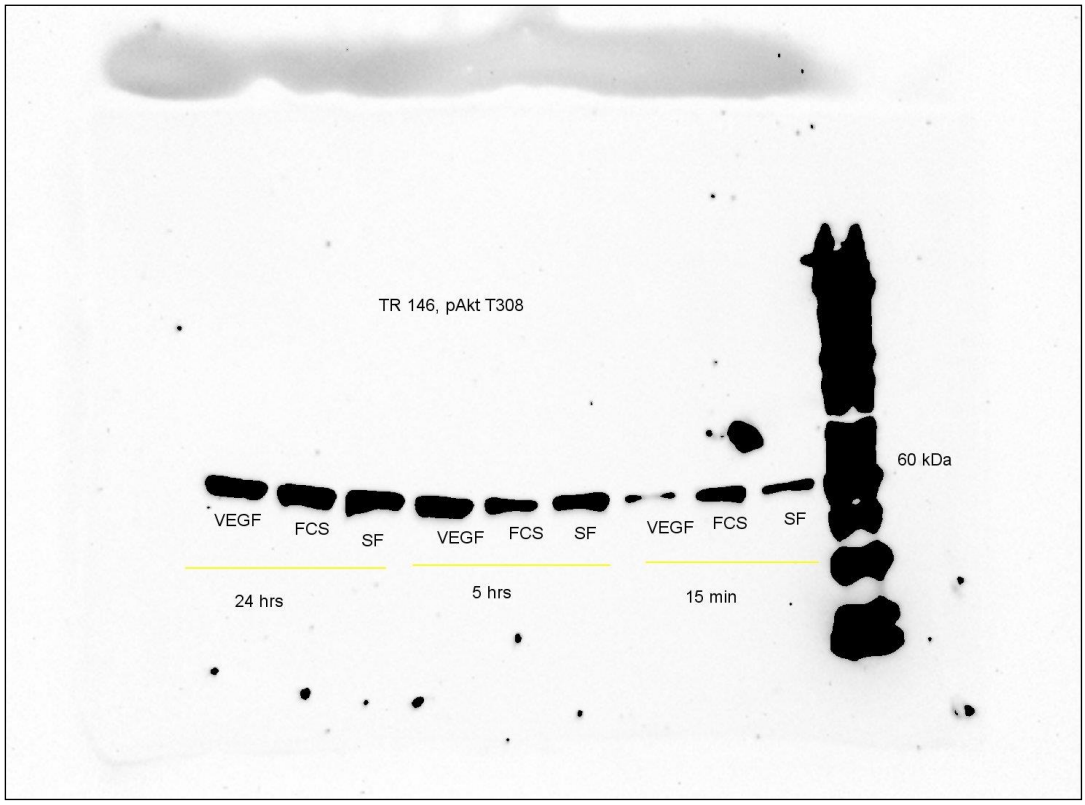
1. anti pAKT Thr 308 # 2965 (Cell Signalling Technology Inc.). Diluted 1:1000 in TBST + 1% (w/v) dried milk (N.B. 5% w/v BSA was recommended by the manufacturer)
2. anti pAKT Ser 473 # 4060 (Cell Signalling Technology Inc.). Diluted 1:2000 in TBST + 1% (w/v) dried milk (N.B. 5% w/v BSA was recommended by the manufacturer)

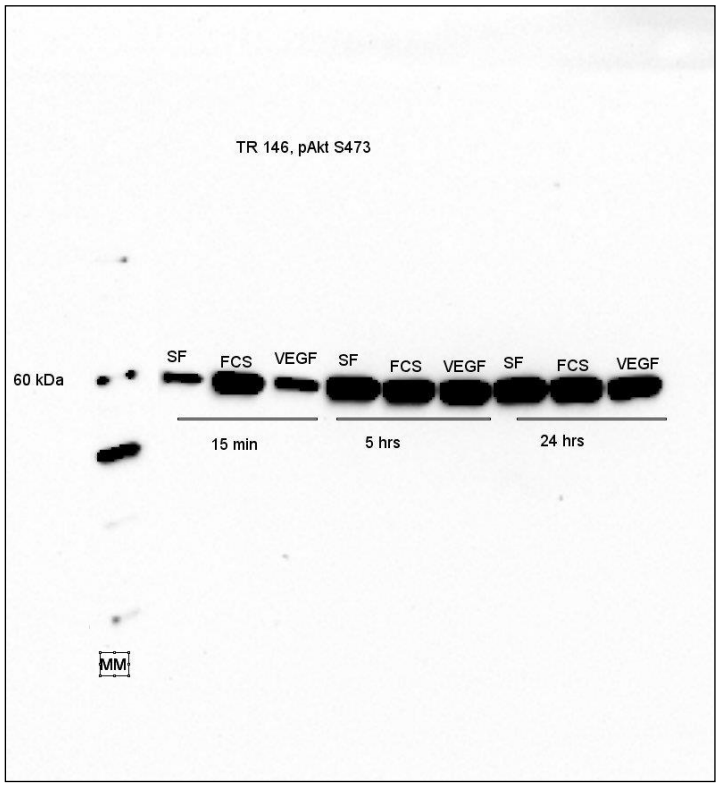
SECONDARY ANTIBODY

1. Goat anti-rabbit HR #7074L (Cell signaling) was used for at a 1:2,000 diluted in 1% (w/v) milk TBST .Washed with TBST.

GEL 1 LOADING			
Lane		µl	
1	MM	2	Western Blot Anti pAkt T308 1:1000 Biochem lab protocol
2	TR146 SF 15 min	15	
3	TR146 FCS 15 min	15	
4	TR146 10n VEGF 15 min	15	
5	TR146 SF 5 hrs	15	
6	TR146 FCS 5 hrs	15	
7	TR146 10n VEGF 5 hrs	15	
8	TR146 SF 24 hrs	15	
9	TR146 FCS 24 hrs	15	
10	TR146 10n VEGF 24 hrs	15	

GEL 2 LOADING			
Lane		µl	
1	MM	2	Western Blot Anti pAkt S473 1:2000 Biochem lab protocol
2	TR146 SF 15 min	15	
3	TR146 FCS 15 min	15	
4	TR146 VEGF 15 min	15	
5	TR146 SF 5 hrs	15	
6	TR146 FCS 5 hrs	15	
7	TR146 VEGF 5 hrs	15	
8	TR146 SF 24 hrs	15	
9	TR146 FCS 24 hrs	15	
10	TR146 VEGF 24 hrs	15	




TR 146, pAkt S473
60 kDa
SF
FCS
VEGF
SF
FCS
VEGF
SF
FCS
VEGF
15 min
5 hrs
24 hrs
MM

Appendix 4. Representative WB experiment report (TR146, VEGF±LY, VEGF±PI103, pAkt S473, pAkt T308)

DATE: 09.10.13	Reducing 'Any kD' SDS PAGE BioRad Pre-cast Gels

EXPERIMENTS

Analysis of cell pAkt ser 473 and pAkt T308. Cells were grown up (~confluent TR146 80%) in 60mm dishes and then transferred to serum free (SF) medium overnight, prior to the experiment. Cells were then transferred to the test conditions (i.e. SF, VEGF, inhibitors) for 15 minutes. Cells were then washed with ice-cold PBS and lysed in RIPA buffer containing protease inhibitor.

SAMPLE PREPARATION FOR GELS

Cells were frozen at -20ºC. Prior to use, the lysates were thawed and then spun at 13,000rpm for 5 minutes. Samples were then mixed with an equal volume of Laemmli sample loading buffer (BioRAD) including 5% (v/v 2-mercaptoethanol). Samples were heated at 95°C for 5 minutes.

WESTERN BLOTTING

As described in method, onto nitrocellulose using transfer buffer. 15V 42 minutes.

PRIMARY ANTIBODIES

1. anti pAKT Ser 473 # 4060 (Cell Signalling Technology Inc.). Diluted 1:2000 in TBST + 1% (w/v) dried milk (N.B. 5% w/v BSA was recommended by the manufacturer)
2. anti pAKT Thr 308 # 2965s (Cell Signalling Technology Inc.). Diluted 1:1000 in TBST + 1% (w/v) dried milk (N.B. 5% w/v BSA was recommended by the manufacturer)

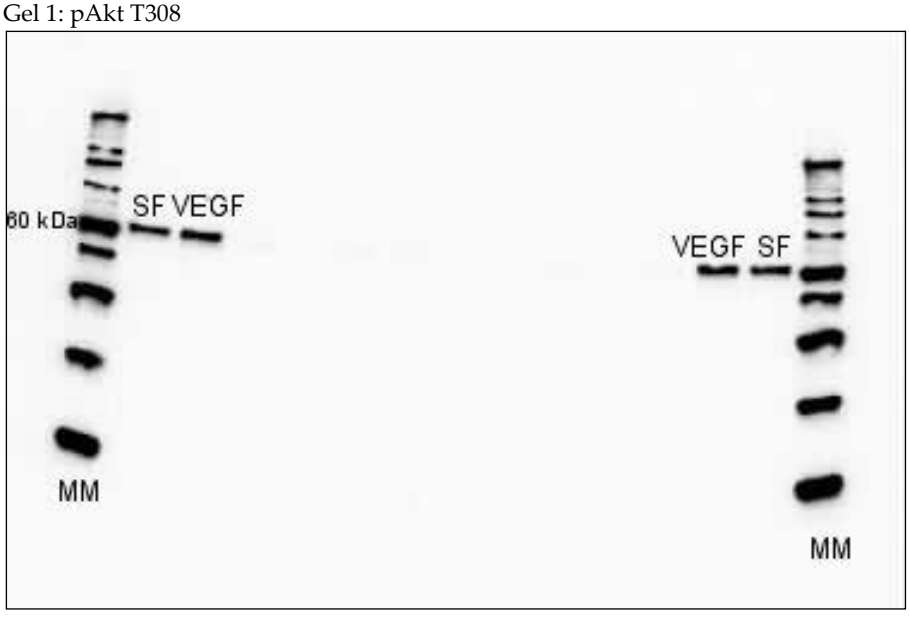
SECONDARY ANTIBODY

1. Goat anti-rabbit HR #7074L (Cell signaling) was used for at a 1:2,000 diluted in 1% (w/v) milk TBST .Washed with TBST.

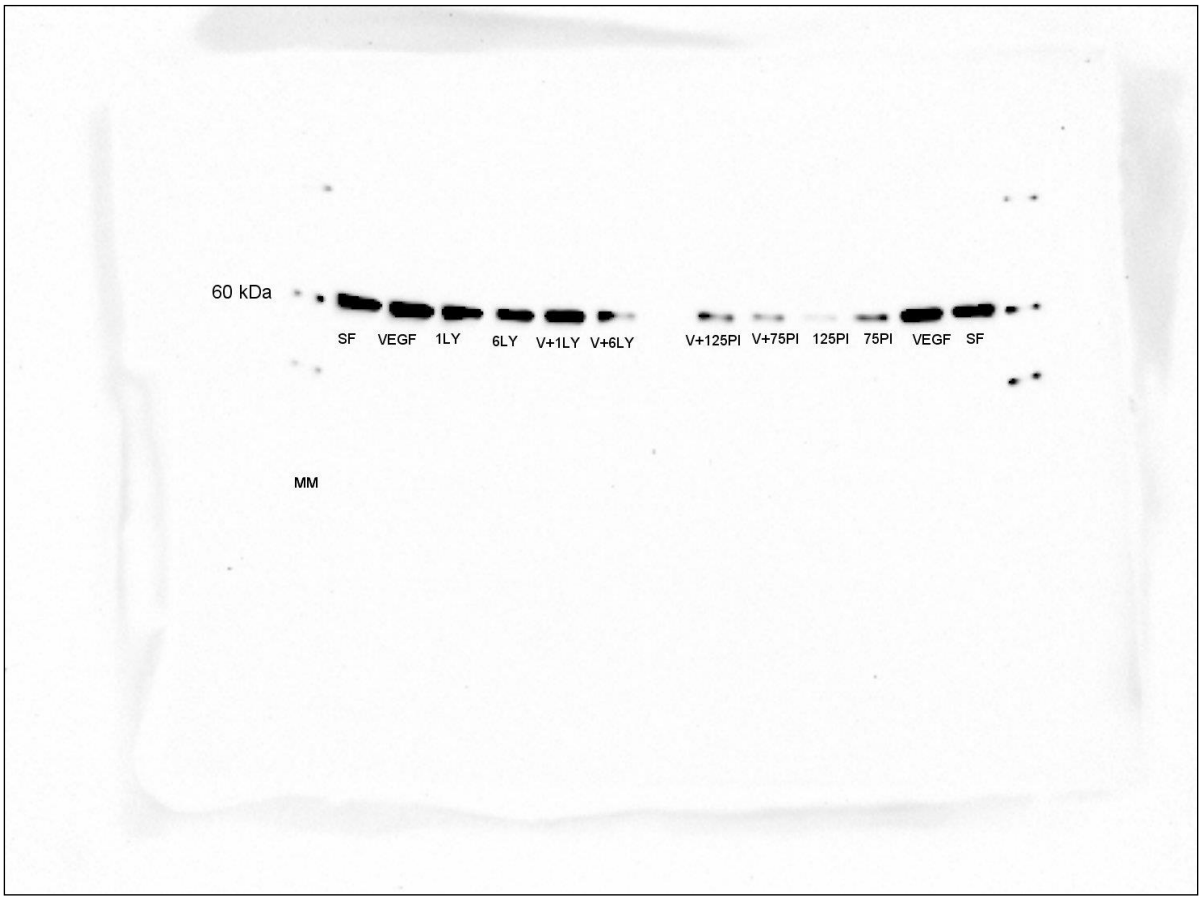
GEL 1 LOADING			
Lane		µl	
1	MM	2	Western Blot Anti pAkt Thr 308 1:1000 Biochem lab protocol
2	TR146 SF	15	
3	TR146 10ng/ml VEGF	15	
4	TR146 1µM LY	15	
5	TR146 6µM LY	15	
6	TR146 VEGF + 1µM LY	15	
7	TR146 VEGF + 6µM LY	15	
8			
9	TR146 VEGF+125nM PI103	15	
10	TR146 VEGF+ 75nM PI103	15	
11	TR146 125nM PI103	15	
12	TR146 75nM PI103	15	
13	TR146 10ng/ml VEGF	15	
14	TR146 SF	15	
15	MM	2	

GEL 2 LOADING			
Lane		µl	
1	MM	2	Western Blot Anti pAkt S473 1:2000 Biochem lab protocol
2	TR146 SF	15	
3	TR146 10ng/ml VEGF	15	
4	TR146 1µM LY	15	
5	TR146 6µM LY	15	
6	TR146 VEGF + 1µM LY	15	
7	TR146 VEGF + 6µM LY	15	
8			
9	TR146 VEGF+125nM PI103	15	
10	TR146 VEGF+ 75nM PI103	15	
11	TR146 125nM PI103	15	
12	TR146 75nM PI103	15	
13	TR146 10ng/ml VEGF	15	
14	TR146 SF	15	
15	MM	2	

Gel 1: pAkt T308



Gel 2: pAkt S473



Appendix 5. Representative WB report (TR146, HaCaT, pPTEN)

DATE: 26.11.13	Reducing 7.5% Mini Protean TGX SDS PAGE BioRad Pre-cast Gels

EXPERIMENTS

Analysis of cell pPTEN (Ser 380) . Cells were grown up (~confluent HaCaT and TR146 80%) in 60mm dishes and then transferred to serum free (SF) medium overnight, prior to the experiment. Cells were then transferred to the test conditions (i.e. SF, VEGF) for 15 minutes, 5 hrs and 24 hrs. Cells were then washed with ice-cold PBS and lysed in RIPA buffer containing protease inhibitor.

SAMPLE PREPARATION FOR GELS

Cells were frozen at -20ºC. Prior to use, the lysates were thawed and then spun at 13,000rpm for 5 minutes. Samples were then mixed with an equal volume of Laemmli sample loading buffer (BioRAD) including 5% (v/v 2-mercaptoethanol). Samples were heated at 95°C for 5 minutes.

WESTERN BLOTTING

As described in method, onto nitrocellulose using transfer buffer. 15V 42 minutes.

PRIMARY ANTIBODIES

1.pPTEN antibody (#9552, Cell Signalling Tech. Inc) was used at 1:1000 diluted in 1% (w/v) milk TBST

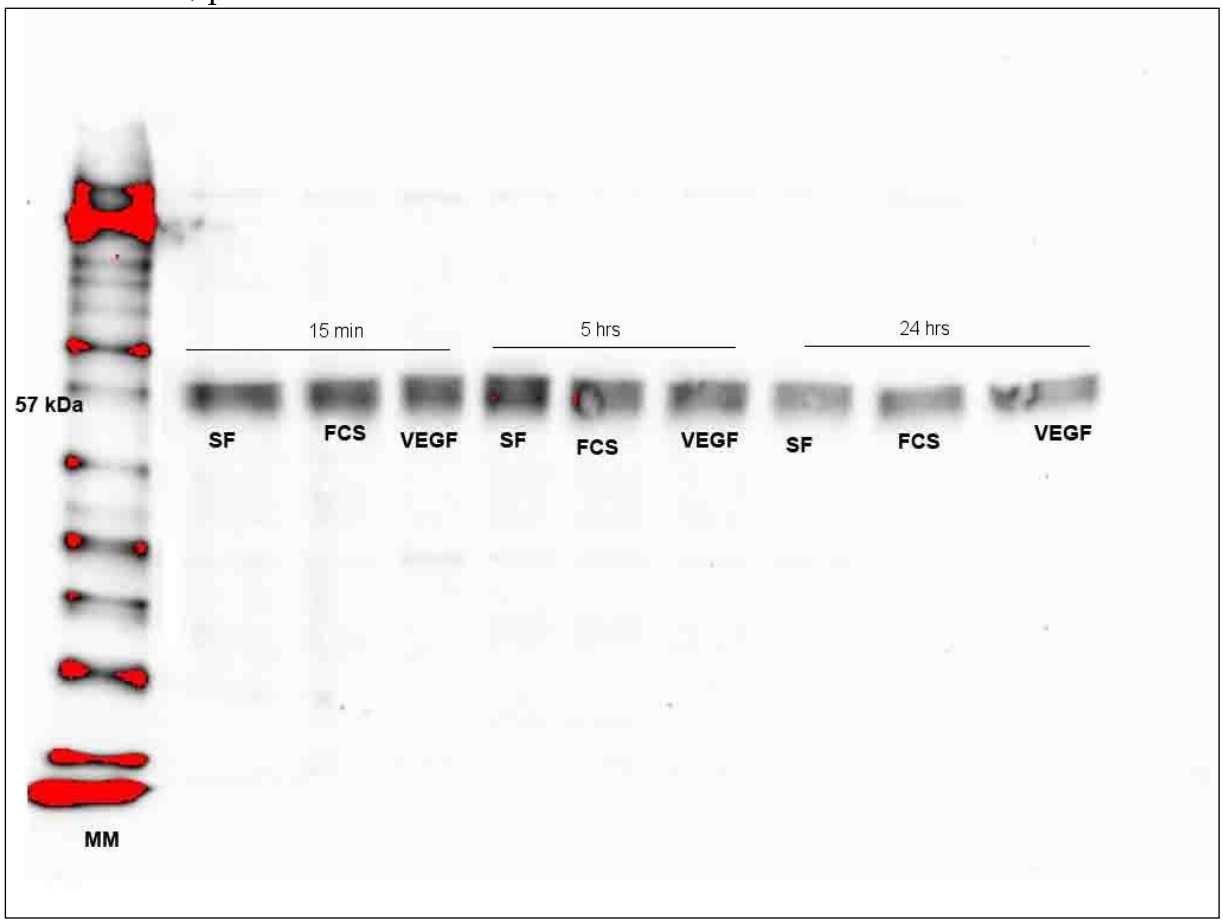
SECONDARY ANTIBODY

1. Goat anti-rabbit HR #7074L (Cell signaling) was used at a 1:2,000 diluted in 1% (w/v) milk TBST .Washed with TBST.

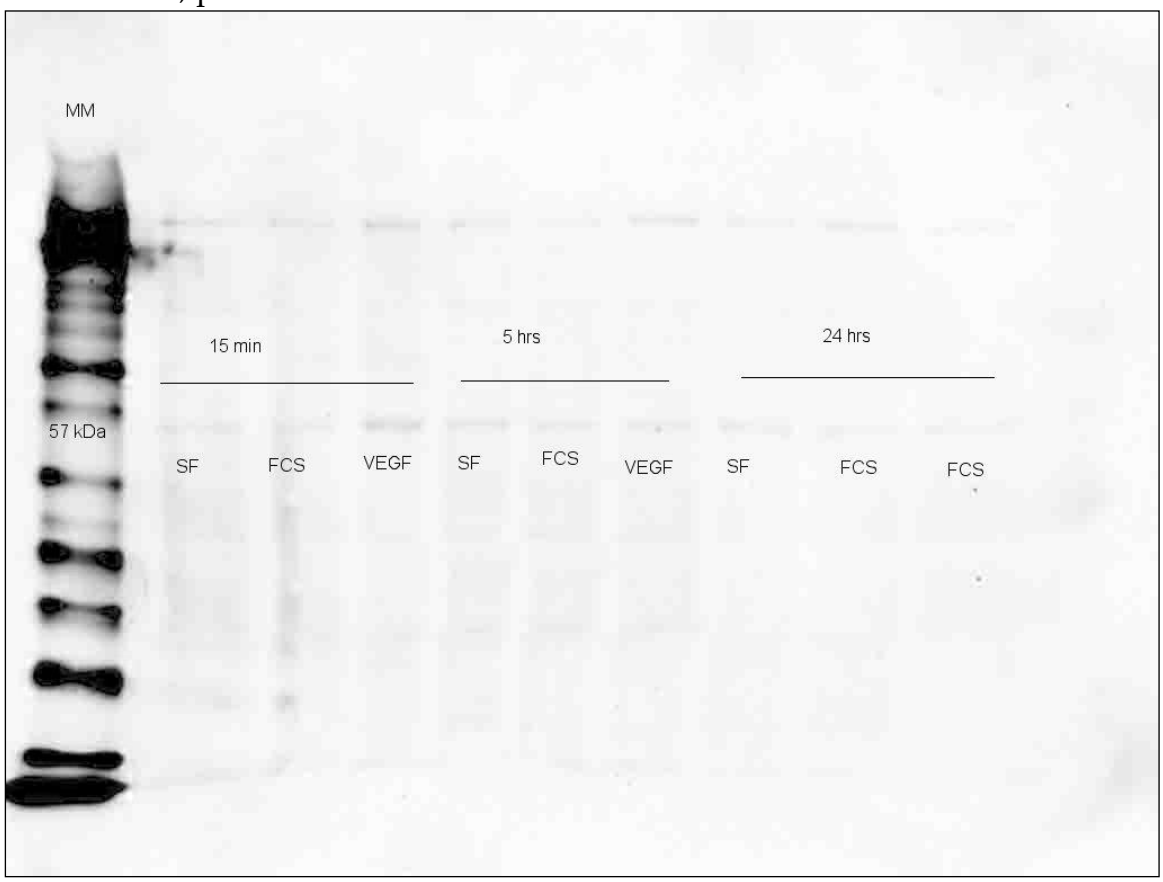
GEL 1 LOADING			
Lane		µl	
1	MM	2	Western Blot Anti pPTEN antibody 1:1000 Biochem lab protocol
2	HacaT SF 15 min	30	
3	HaCaT FCS 15 min	30	
4	HaCaT VEGF 15 min	30	
5	HacaT SF 5hrs	30	
6	HaCaT FCS 5hrs	30	
7	HaCaT VEGF 5hrs	30	
8	HacaT SF 24 hrs	30	
9	HaCaT FCS 24 hrs	30	
10	HaCaT VEGF 24 hrs	30	

GEL 2 LOADING			
Lane		µl	
1	MM	2	Western Blot Anti pPTEN antibody 1:1000 Biochem lab protocol
2	TR146 SF 15 min	30	
3	TR146 FCS 15 min	30	
4	TR146 VEGF 15 min	30	
5	TR146 SF 5hrs	30	
6	TR146 FCS 5hrs	30	
7	TR146 VEGF 5hrs	30	
8	TR146 SF 24 hrs	30	
9	TR146 FCS 24 hrs	30	
10	TR146 VEGF 24 hrs	30	

Gel 1: HaCaT, pPTEN

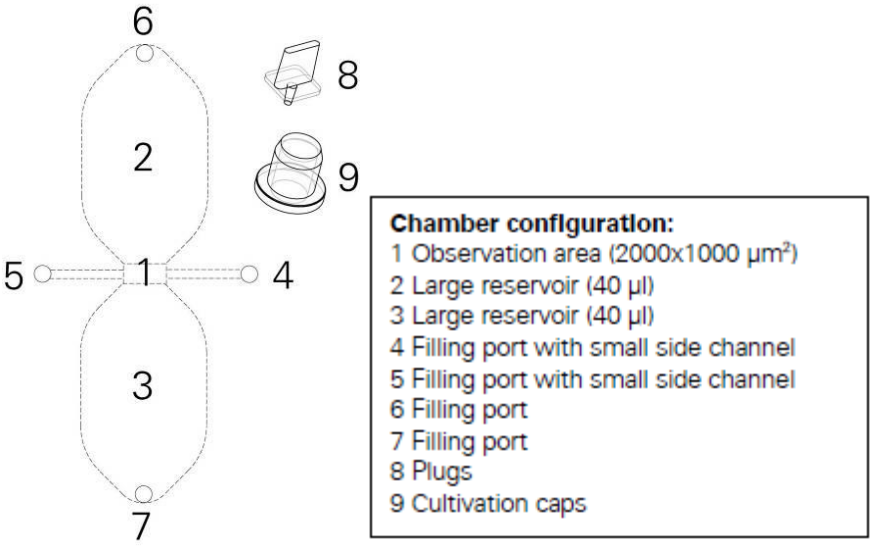


Gel 2: TR146, pPTEN



Appendix 6. 2D Chemotaxis assay

To observe the chemotactical responses of adherent oral cancer cell the μ-slide chemotaxis 2D (IBIDI GmbH) was used. Chemotactical movement requires the linear concentration profile which is generated in this slide by diffusion and stable for 48 hours. Two reservoirs in the slide contain different chemoattractant concentration and are connected by a thin slit. The configuration of this slide is given below:



Materials required:

1. Adherent cells: HaCaT and TR-146
2. Chemoattractant (VEGF, serum free medium)
3. Tissue culture treated μ-slide chemotaxis 2D (80306)
4. Heated stage
5. A humid chamber (90mm petri dish with wet tissue)
6. Inverted microscope (Olympus IX70)
7. Time lapse video equipment (CCD camera, acquisition software)

8. Beveled pipette tip
9. Slant cosmetic tweezers

Method

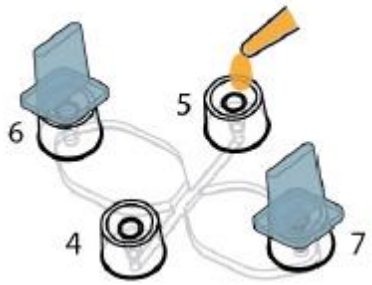
1. Seeding cells

a) All the media and μ-slide and plugs were placed into the incubator the day before conducting the experiment. μ-slide was placed in a humid chamber to reduce the evaporation.

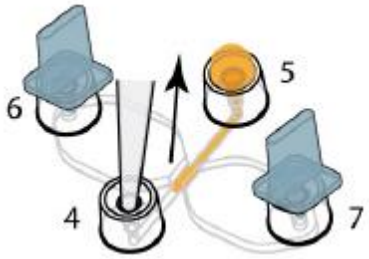
b) Cell suspension of 3×10^6 cells/ml concentration was then made and used for this experiment.

c) Filling ports 6 & 7 were then closed by plugs.

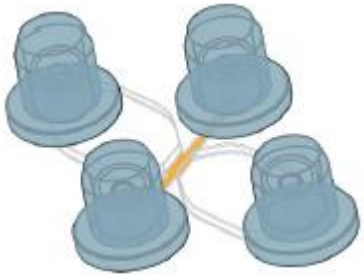
d) 6 μl of cell suspension was applied on top of filling port 5.



e) Air was then gently aspirated from the opposite filling port 4 using the same pipette settings. The cell suspension on top of port 5 was flushed inside filling the entire observation area homogeneously.



f) Plugs from filling port 6 & 7 were then removed gently and all the ports were covered with cultivation caps.

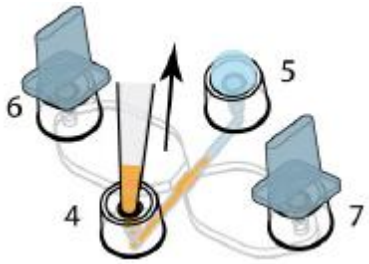


g) The slide with cell suspension was then placed in a humid chamber and incubated for 4-5 hours until cell attachment.

2. Cell attachment

a) After cell attachment, all the cultivation caps were removed and filling port 6 & 7 were closed by plugs.

b) 10 µl cell free medium was then placed onto filling port 5 and the same amount of was aspirated from filling port 4.

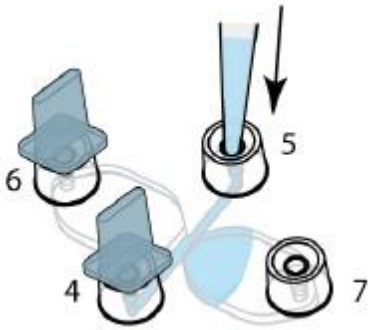


c) This step of washing was repeated twice.

3. Filling the reservoirs

a) Plug from filling port 7 was then transferred to port 4.

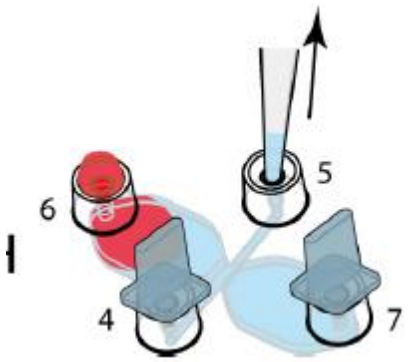
b) 45 µl cell-free medium was then gently injected into port 5 to fill one reservoir. Medium was injected very slowly until port 7 was completely filled.



c) The Plug from port 6 was then transferred to port 7. The other reservoir then filled by injecting 45 µl cell-free medium into port 5.

4. Applying chemoattractant

a) 18 µl of chemoattractant was applied onto port 6 and the same amount of liquid was aspirated from port 5.



b) Filling port 6 & 7 both were then closed by plugs.

5. Video microscopy

a) Slide with attached cells was then mounted in the heated stage on the microscope.

b) A 4X objective lens was used to observe the cell movement for 24 hours. Time-lapse images capturing was set for every 15 minutes.

c) After 24 hours images were exported as uncompressed single page .tif files.

Appendix 7. Tracking cells and analysing chemotaxis

ImageJ plugin, 'Manual tracking' was used to track the cells after the chemotaxis experiment. This plugin was able to determine the movement of objects between frames of a sequential stack. At least 30 cells in the observation field were tracked. Tracking of cells with Manual tracking Plugin was done as follows:

1. ImageJ was opened and a movie as single page .tif files was imported by using File/import/image sequence.
2. 'Manual tracking' plugin was then opened.
3. 'Add track' was selected.
4. First cell was then followed by clicking on the cell's midpoint, through all the time points. The software created a data table after clicking first cells. The data table contains all tracked cells and time points with (x,y) positions.
5. Data table was then saved after tracking is completed.
6. Image sequences were also saved as a movie file (.avi).

Tracked data was then analysed by IBIDI's chemotaxis and migration tool. The detailed procedures followed as below:

1. Data table from manual tracking was imported in chemotaxis tool software.
2. Number of slices (number of pictures used for tracking) were then selected.
3. The software was then calibrated by setting x/y calibration (length of one pixel in μm) and the time interval (15 min).
4. 'Apply settings' was then pressed.

5. A trajectory plot was created and exported as an image file.

6. The values for centre of mass, FMI and Rayleigh test were then exported.

Appendix 8. Boyden Chamber: HacaT and $VEGF_{121}$

BD-Assay of HacaT and $VEGF_{121}$

Exp. Code: MI 030511aHacaTVEGF$_{121}$

Aim: To compare the effect of $VEGF_{121}$ on the migration of normal human Keratinocyte, HacaT in the Boyden chamber.

Cells: Normal Human Keratinocyte, HacaT
Cells: semi-confluent, 5.68 x10^5cells/90mm dish
Cells have been grown on 90 mm dishes in 10% FCS-MEM-Glu media
Last farmed 1 day before the experiment on 03.05.11

Protocol:
$VEGF_{121}$: Stock concentration 100µg/ml, Code no 10-1296C (Insight Biotechnology Ltd.UK) tested at final concentrations of 1 pg/ml, 10 pg/ml, 100pg/ml, 1ng/ml, 10ng/ml and 100 ng/ml.
Native Collagen: 100 µg/ml.
Upper chamber: Cells in SF-BSA (2 µg/ml);
5.0x10^4 cells/50µl/well (1.0 x 10^6 cells/ml)
Filter: 8 µm pore filters coated with native collagen, 8°C O/N, Collagen used 1st time.
Lower chamber: with SF-BSA (2 µg/ml)

SF-BSA	10 pg/ml $VEGF_{121}$	1 ng/ml $VEGF_{121}$	100 ng/ml $VEGF_{121}$
1 pg/ml $VEGF_{121}$	100 pg/ml $VEGF_{121}$	10 ng/ml $VEGF_{121}$	1ng/ml EGF

Incubation time: 5 hours
Staining time: Overnight

Results:
Chamber: 5 hours' incubation
x 200 (Objective x10, eye piece x20)

	Well	Migrated cell no.			Mean	Stdev
	1	16	12	11	13	2.645751
	2	13	14	12	13	1
SF-BSA	3	16	13	11	13.33333	2.516611
5hr	4	15	13	14	14	1
Nat.col	5	16	14	15	15	1
8µm	6	15	12	13	13.33333	1.527525
Mean					**13.61+/-0.77**	
1pg/ml $VEGF_{121}$	1	14	16	12	14	2
5hr	2	12	14	16	14	2
Nat.col	3	13	12	15	13.33333	1.527525
8µm	4	14	16	19	16.33333	2.516611

	5	13	14	12	13	1
	6	15	17	14	15.33333	1.527525
Mean					**14.33+/-1.32**	
	1	13	15	12	13.33333	1.527525
	2	13	14	12	13	1
10pg/ml $VEGF_{121}$	3	15	17	15	15.66667	1.154701
5hr	4	17	14	16	15.66667	1.527525
Nat.col	5	14	16	13	14.33333	1.527525
8µm	6	11	15	12	12.66667	2.081666
Mean					**14.11+/-1.32**	
	1	13	16	15	14.66667	1.527525
	2	17	14	12	14.33333	2.516611
100pg/ml $VEGF_{121}$	3	11	14	13	12.66667	1.527525
5hr	4	12	11	14	12.33333	1.527525
Nat.col	5	18	12	13	14.33333	3.21455
8µm	6	16	14	11	16	2.516611
Mean					**14.05+/-1.35**	
	1	15	16	14	15	1
	2	17	12	15	14.66667	2.516611
1ng/ml $VEGF_{121}$	3	14	13	12	13	1
5hr	4	15	18	14	15.66667	2.081666
Nat.col	5	13	12	11	12	1
8µm	6	15	16	13	14.66667	1.527525
Mean					**14.16+/-1.37**	
	1	18	16	15	16.33333	1.527525
	2	16	14	13	14.33333	1.527525
10 ng/ml $VEGF_{121}$	3	14	17	12	14.33333	2.516611
5hr	4	11	13	16	13.33333	2.516611
Nat.col	5	13	16	17	15.33333	2.081666
8µm	6	13	15	18	15.33333	2.516611
Mean					**14.83+/-1.04**	
	1	11	13	10	11.33333	1.527525
	2	15	16	14	15	1
100ng/ml $VEGF_{121}$	3	17	16	15	16	1
5hr	4	14	16	18	16	2
Nat.col	5	12	14	13	13	1
8µm	6	15	17	18	16.66667	1.527525
Mean					**14.66+/-2.07**	
	1	66	72	78	72	6
	2	68	74	73	71.66667	3.21455
1ng/ml EGF	3	72	75	77	74.66667	2.516611
5hr	4	82	84	79	81.66667	2.516611
Nat.col	5	70	69	78	72.33333	4.932883
8µm	6	72	78	75	75	3
Mean					**74.55+/-3.75**	

Summary of Data:

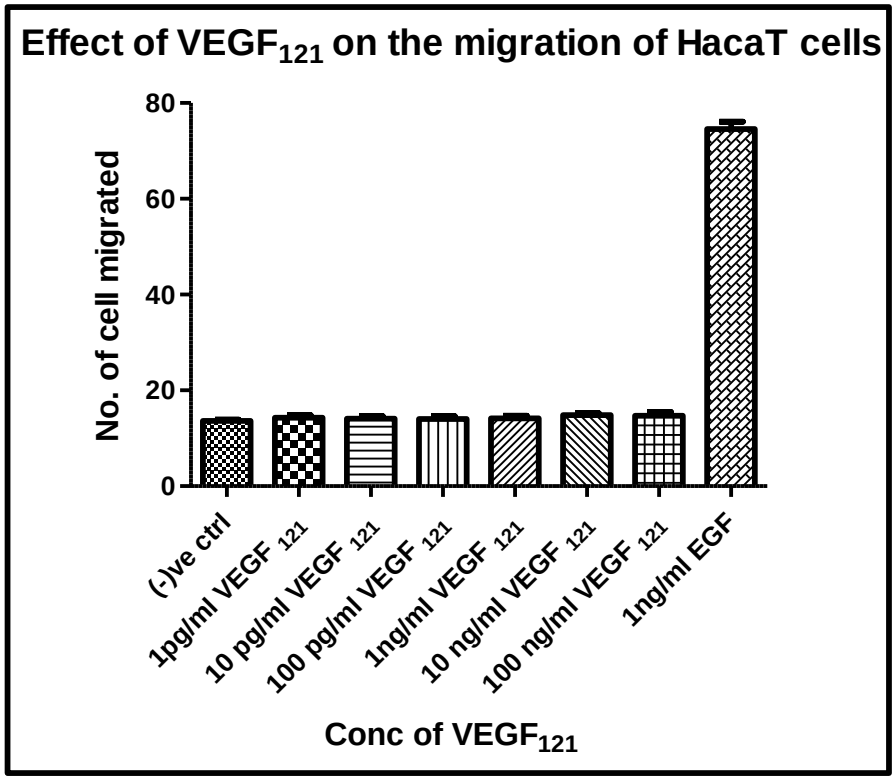
Chamber 1: HacaT with $VEGF_{121}$

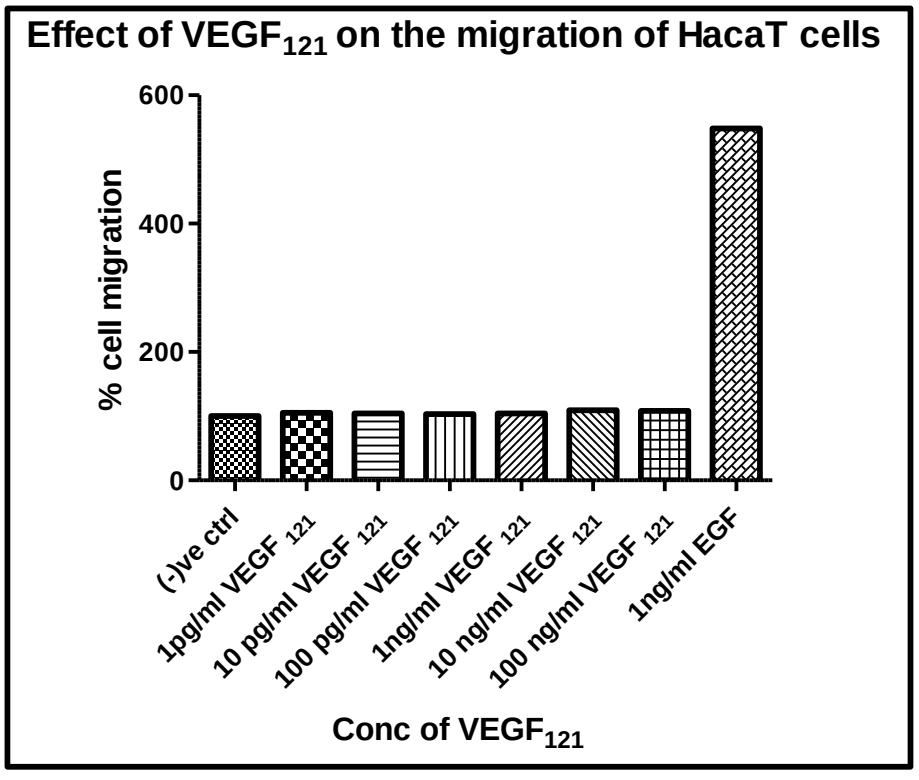
5 hours incubation

8μm pore filter coated with native collagen

Base medium: SF-BSA(2 μg/ml)

	Mean	SD	% migration	Significance?	
				Bonferroni's (P<0.05)	Kruskal-Wallis & Dunnet's test (p<0.05)
SF-BSA	13.61	+/-0.77	100		
1pg/ml $VEGF_{121}$	14.33	+/- 1.26	105	N	N
10pg/ml $VEGF_{121}$	14.11	+/-1.32	104	N	N
100pg/ml $VEGF_{121}$	14.05	+/-1.35	103	N	N
1ng/ml $VEGF_{121}$	14.16	+/-1.37	104	N	N
10ng/ml $VEGF_{121}$	14.83	+/-1.04	109	N	N
100ng/ml $VEGF_{121}$	14.66	+/-2.07	108	N	N
1ng/ml EGF	74.55	+/-3.75	548	Y	Y


Effect of VEGF121 on the migration of HacaT cells
No. of cell migrated
80
60
40
20
0
(-)ve ctrl
1pg/ml VEGF 121
10 pg/ml VEGF 121
100 pg/ml VEGF 121
1ng/ml VEGF 121
10 ng/ml VEGF 121
100 ng/ml VEGF 121
1ng/ml EGF
Conc of VEGF121


Effect of VEGF121 on the migration of HacaT cells
% cell migration
600
400
200
0
(-)ve ctrl
1pg/ml VEGF 121
10 pg/ml VEGF 121
100 pg/ml VEGF 121
1ng/ml VEGF 121
10 ng/ml VEGF 121
100 ng/ml VEGF 121
1ng/ml EGF
Conc of VEGF121

Appendix 9. Boyden Chamber: TYS and $VEGF_{121}$

BD-Assay of TYS and $VEGF_{121}$

Exp. Code: MI 06.12.10bTYSVEGF$_{121}$

Aim: To compare the effect of $VEGF_{121}$ on the migration of Oral tumour cell line, TYS in the Boyden chamber.

Cells: Oral adeno-carcinoma cell line, TYS
Cells: semi-confluent, $4.8x10^5$cells/90mm dish
Cells have been grown on 90 mm dishes in 15% DCS-MEM-Glu media
Last farmed 1 day before the experiment on 06.12.10

Protocol:
$VEGF_{121}$: Stock concentration 100μg/ml, Code no 10-1296C (Insight Biotechnology Ltd.UK) tested at final concentrations of 1 pg/ml, 10 pg/ml, 100pg/ml, 1ng/ml, 10ng/ml and 100 ng/ml.
Native Collagen: 100 μg/ml.
Upper chamber: Cells in SF-BSA (2 μg/ml);
$5.0x10^4$ cells/50μl/well (1.0×10^6 cells/ml)
Filter: 8 μm pore filters coated with native collagen, 8°C O/N, Collagen used 2nd time.
Lower chamber: with SF-BSA (2 μg/ml)

SF-BSA(1)	10 pg/ml $VEGF_{121}$	1 ng/ml $VEGF_{121}$	100 ng/ml $VEGF_{121}$
1 pg/ml $VEGF_{121}$	100 pg/ml $VEGF_{121}$	10 ng/ml $VEGF_{121}$	SF-BSA(2)

Incubation time: 5 hours
Staining time: Overnight

Results:
Chamber: 4 hours' incubation
x 200 (Objective x10, eye piece x20)

	Well	Migrated cell no.			Mean	Stdev
SF-BSA(1) 5hr Nat.col 8μm	1	23	25	27	25	2
	2	25	26	26	25.66667	0.57735
	3	24	20	22	22	2
	4	25	21	20	22	2.645751
	5	25	22	23	23.33333	1.527525
	6	23	27	29	26.33333	3.05505
Mean					**24.05+/-0.86**	
1pg/ml VEGF121 5hr Nat.col	1	37	36	34	35.66667	1.527525
	2	32	29	31	30.66667	1.527525
	3	29	27	25	27	2

5µm	4	36	35	33	34.66667	1.527525
	5	33	29	31	31	2
	6	30	36	33	33	3
Mean					**32.00+/-0.57**	
10pg/ml VEGF121 5hr Nat.col 8µm	1	33	36	39	36	3
	2	45	42	43	43.33333	1.527525
	3	42	38	44	41.33333	3.05505
	4	40	49	46	45	4.582576
	5	42	39	43	41.33333	2.081666
	6	38	44	47	43	4.582576
Mean					**41.66+/-1.25**	
100pg/ml VEGF121 5hr Nat.col 8µm	1	42	43	47	44	2.645751
	2	52	45	49	48.66667	3.511885
	3	48	45	44	45.66667	2.081666
	4	49	47	45	47	2
	5	50	47	46	47.66667	2.081666
	6	52	51	49	52	1.527525
Mean					**47.5+/-0.68**	
1ng/ml VEGF121 5hr Nat.col 8µm	1	52	48	53	51	2.645751
	2	56	58	54	56	2
	3	58	52	55	55	3
	4	54	56	52	54	2
	5	49	52	56	52.33333	3.511885
	6	48	52	51	50.33333	2.081666
Mean					**53.11+/-0.62**	
10 ng/ml VEGF121 5hr Nat.col 8µm	1	53	49	56	52.66667	3.511885
	2	52	50	48	50	2
	3	56	56	54	55.33333	1.154701
	4	66	62	64	64	2
	5	59	62	63	61.33333	2.081666
	6	54	58	55	55.66667	2.081666
Mean					**56.5+/-0.76**	
100ng/ml VEGF121 5hr Nat.col 8µm	1	32	35	30	32.33333	2.516611
	2	29	25	31	28.33333	3.05505
	3	26	32	29	29	3
	4	25	28	24	25.66667	2.081666
	5	32	27	30	29.66667	2.516611
	6	26	29	32	29	3
Mean					**29.00+/-0.38**	
SF-BSA(2) 5hr	1	15	18	21	18	3
	2	17	19	23	19.66667	3.05505

Nat.col 8μm	3	20	22	16	19.33333	3.05505
	4	24	21	19	21.33333	2.516611
	5	23	18	16	19	3.605551
	6	19	21	24	21.33333	2.516611
Mean					**19.77+/-0.40**	

Summary of Data:

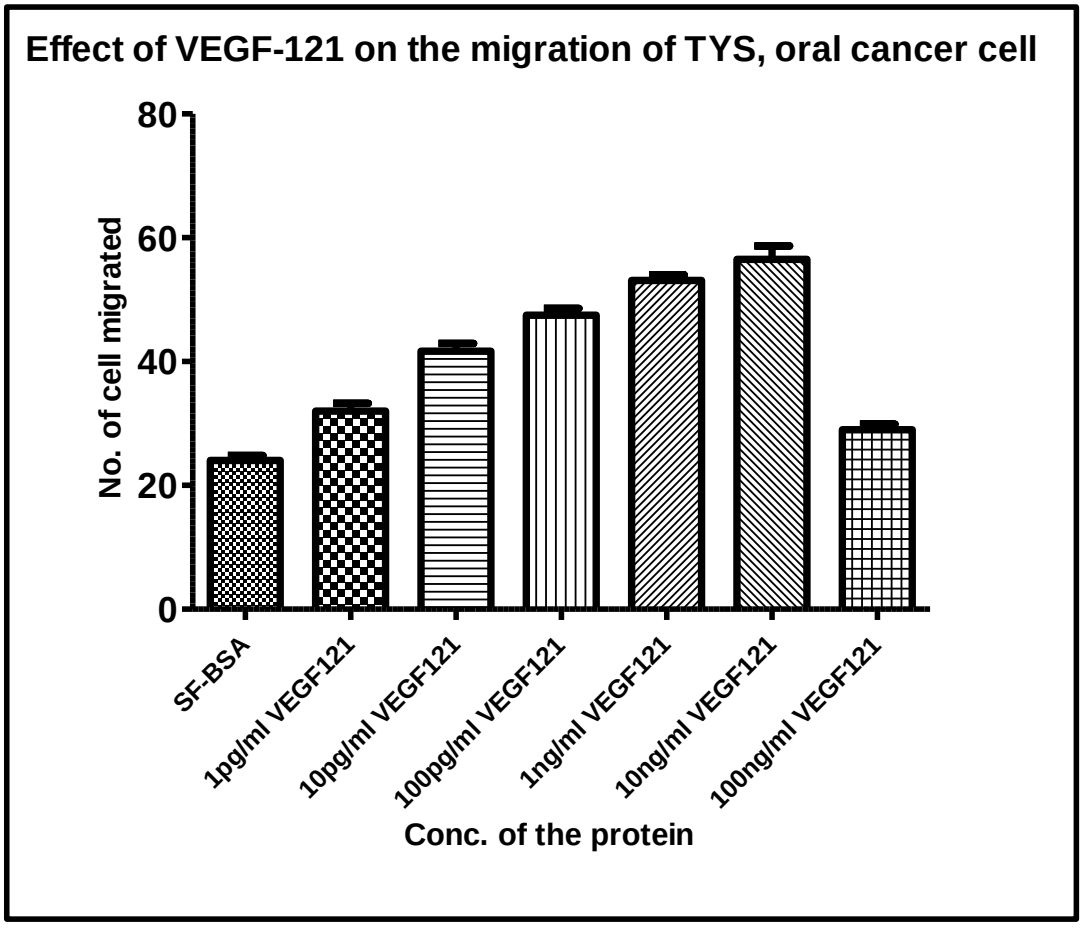
Chamber 1: TYS with $VEGF_{121}$

5 hours incubation

8μm pore filter coated with native collagen

Base medium: SF-BSA(2 μg/ml)

	Mean	**SD**	**% migration**	**Significance?**	
				Bonferroni's (P<0.05)	**Kruskal-Wallis & Dunnet's test (p<0.05)**
SF-BSA(1)	24.55	+/-0.86			
SF-BSA(2)	19.77	+/-0.40			
SF-BSA	22.16		100		
1pg/ml $VEGF_{121}$	32.00	+/ 0.57	144	Y	N
10pg/ml $VEGF_{121}$	41.66	+/-1.25	188	Y	N
100pg/ml $VEGF_{121}$	47.50	+/-0.68	214	Y	Y
1ng/ml $VEGF_{121}$	53.11	+/-0.62	240	Y	Y
10ng/ml $VEGF_{121}$	56.50	+/-0.76	255	Y	Y
100ng/ml $VEGF_{121}$	29.00	+/-0.38	131	N	N


Effect of VEGF-121 on the migration of TYS, oral cancer cell
No. of cell migrated
80
60
40
20
0
SF-BSA
1pg/ml VEGF121
10pg/ml VEGF121
100pg/ml VEGF121
1ng/ml VEGF121
10ng/ml VEGF121
100ng/ml VEGF121
Conc. of the protein

Appendix 10. Boyden Chamber: COM D25 and $VEGF_{121}$

BD-Assay of COM D25 and $VEGF_{121}$

Exp. Code: MI 11.09.12aCOM D25$VEGF_{121}$

Aim: To compare the effect of $VEGF_{121}$ on the migration of mouth cancer associated fibroblast cell , COM D25 in the Boyden chamber.

Cells: Mouth cancer associated fibroblast cell line, COM D25
Cells: semi-confluent, 2.5 $x10^5$cells/90mm dish
Cells have been grown on 90 mm dishes in 10% FCS-MEM-Glu media
Last farmed 1 day before the experiment on 11.09.12

Protocol:
$VEGF_{121}$: Stock concentration 100μg/ml, Code no 10-1296C (Insight Biotechnology Ltd.UK) tested at final concentrations of 1 pg/ml, 10 pg/ml, 100pg/ml, 1ng/ml, 10ng/ml and 100 ng/ml.
Native Collagen: 100 μg/ml.
Upper chamber: Cells in SF-BSA (2 μg/ml);
$5.0x10^4$ cells/50μl/well (1.0×10^6 cells/ml)
Filter: 8 μm pore filters coated with native collagen, 8°C O/N, Collagen used 1st time.
Lower chamber: with SF-BSA (2 μg/ml)

SF-BSA(1)	10 pg/ml $VEGF_{121}$	1 ng/ml $VEGF_{121}$	100 ng/ml $VEGF_{121}$
1 pg/ml $VEGF_{121}$	100 pg/ml $VEGF_{121}$	10 ng/ml $VEGF_{121}$	SF-BSA(2)

Incubation time: 5 hours
Staining time: Overnight

Results:
Chamber: 5 hours' incubation
x 200 (Objective x10, eye piece x20)

	Well	**Migrated cell no.**			**Mean**	**Stdev**
SF-BSA(1) 5hr Nat.col 8μm	1	16	14	18	16	2
	2	15	19	14	16	2.645751
	3	14	17	19	16.66667	2.516611
	4	20	22	18	20	2
	5	18	22	17	19	2.645751
	6	19	22	16	19	3
Mean					**17.77**	**0.39**
1pg/ml VEGF121	1	21	15	19	18.33333	3.05505

5hr Nat.col 5µm	2	26	20	24	23.33333	3.05505
	3	20	29	23	24	4.582576
	4	19	24	26	23	3.605551
	5	17	20	24	20.33333	3.511885
	6	36	29	32	32.33333	3.511885
Mean					**23.55**	**0.55**
10pg/ml VEGF121 5hr Nat.col 8µm	1	24	22	26	24	2
	2	29	21	23	24.33333	4.163332
	3	25	20	22	22.33333	2.516611
	4	25	24	20	23	2.645751
	5	26	23	21	23.33333	2.516611
	6	24	21	28	24.33333	3.511885
Mean					**23.56**	**0.79**
100pg/ml VEGF121 5hr Nat.col 8µm	1	26	22	24	24	2
	2	21	20	29	23.33333	4.932883
	3	19	23	22	21.33333	2.081666
	4	26	22	24	24	2
	5	27	28	26	27	1
	6	24	29	22	24	3.605551
Mean					**23.94**	**1.41**
1ng/ml VEGF121 5hr Nat.col 8µm	1	26	29	28	27.66667	1.527525
	2	32	26	24	27.33333	4.163332
	3	26	29	20	25	4.582576
	4	22	28	26	25.33333	3.05505
	5	24	26	27	25.66667	1.527525
	6	26	28	24	26	2
Mean					**26.16**	**1.34**
10 ng/ml VEGF121 5hr Nat.col 8µm	1	19	24	27	23.33333	4.041452
	2	35	29	28	30.66667	3.785939
	3	25	24	29	26	2.645751
	4	25	28	27	26.66667	1.527525
	5	29	24	28	27	2.645751
	6	34	30	29	31	2.645751
Mean					**27.44**	**0.91**
100ng/ml VEGF121 5hr Nat.col 8µm	1	15	19	20	18	2.645751
	2	17	19	18	18	1
	3	16	17	19	17.33333	1.527525
	4	19	22	17	19.33333	2.516611

	5	17	20	19	18.66667	1.527525
	6	20	24	17	20.33333	3.511885
Mean					**18.6**	**0.93**

Summary of Data:

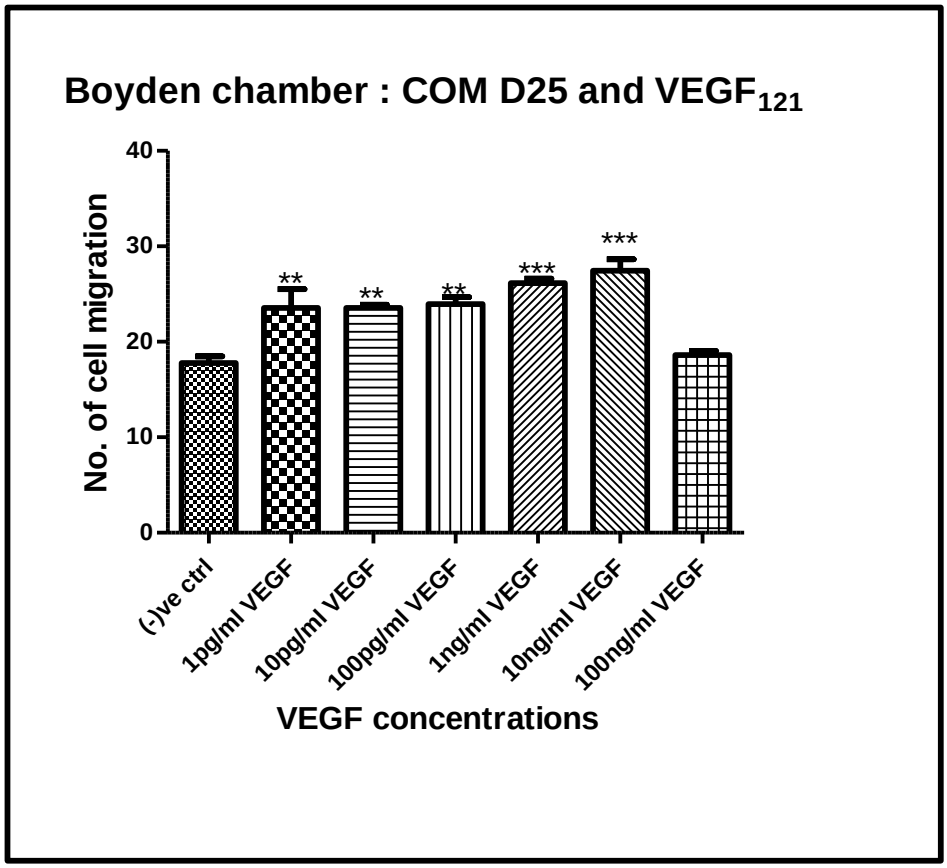
Chamber 1: COM D25 with $VEGF_{121}$

5 hours incubation

8μm pore filter coated with native collagen

Base medium: SF-BSA(2 μg/ml)

	Mean	**SD**	**% migration**	**Significance?**	
				Bonferroni's (P<0.05)	**Kruskal-Wallis & Dunnet's test (p<0.05)**
SF-BSA	17.77	+/-0.39	100		
1pg/ml $VEGF_{121}$	23.55	+/ -0.55	132	Y**	N
10pg/ml $VEGF_{121}$	23.56	+/-0.79	133	Y**	N
100pg/ml $VEGF_{121}$	23.94	+/-1.41	134	Y**	N
1ng/ml $VEGF_{121}$	26.16	+/-1.34	147	Y***	N
10ng/ml $VEGF_{121}$	27.44	+/-0.91	154	Y***	Y
100ng/ml $VEGF_{121}$	18.6	+/-0.93	105	N	Y


Boyden chamber : COM D25 and VEGF121
No. of cell migration
40
30
20
10
0
**
**
**
***
***
(-)ve ctrl
1pg/ml VEGF
10pg/ml VEGF
100pg/ml VEGF
1ng/ml VEGF
10ng/ml VEGF
100ng/ml VEGF
VEGF concentrations

Appendix 11. Boyden Chamber: TYS and VEGF + PI103

BD-Assay of TYS and VEGF+ PI103

Exp. Code: MI 170511aHACATVEGFPI103

Aim: To compare the effect of PI103 (PI3Kinase and mTORC2 inhibitor) on the migration effect of VEGF on the TYS cells in the Boyden chamber.

Cells: TYS, Oral adeno-carcinoma cell line
Cells: semi-confluent, $1.13x10^6$ cells/90mm dish
Cells have been grown on 90 mm dishes in 10% FCS-MEM-Glu media
Last farmed 1 day before the experiment on 17.05.11

Protocol:
PI103: Stock concentration 10mM, Code no 528100 (Merck Chemicals Ltd, UK) tested at final concentrations of 75nm, 125nm and 250nm.
Native Collagen: 100 μg/ml.
Upper chamber: Cells in SF-BSA (2 μg/ml);
$5.0x10^4$ cells/50μl/well (1.0×10^6 cells/ml)
Filter: 8 μm pore filters coated with native collagen, 8°C O/N, Collagen used 1st time.
Lower chamber: with SF-BSA (2 μg/ml)

SF-BSA	125nM PI103	10 ng/ml VEGF+ 75nM PI103	10 ng/ml VEGF+ 250 nM PI103
75nM PI103	250 nMPI103	10 ng/ml VEGF+ 125nM PI103	10ng/ml VEGF

Incubation time: 5 hours
Staining time: Overnight

Results:
Chamber: 5 hours' incubation
x 200 (Objective x10, eye piece x20)

	Well	**Migrated cell no.**			**Mean**	**Stdev**
	1	25	21	23	23	2
	2	25	25	22	24	1.732051
SF-BSA	3	28	30	26	28	2
5hr	4	22	23	24	23	1
Nat.col	5	26	25	23	24.66667	1.527525
8μm	6	24	25	23	24	1
Mean					**24.44+/-1.85**	
	1	17	18	16	17	1
	2	16	17	17	16.66667	0.57735
75nM PI103	3	12	14	13	13	1
5hr	4	17	18	16	17	1
Nat.col	5	16	18	17	17	1
8μm	6	12	17	14	14.33333	2.516611

Mean					**15.83+/-1.73**	
	1	13	11	12	12	1
	2	12	11	10	11	1
125nM PI103	3	10	9	11	10	1
5hr	4	13	12	12	12.33333	0.57735
Nat.col	5	11	12	13	12	1
8µm	6	14	21	17	17.33333	3.511885
Mean					**12.44+/-2.54**	
	1	9	11	10	10	1
	2	11	11	10	10.66667	0.57735
250nM PI103	3	10	9	11	10	1
5hr	4	9	10	10	9.666667	0.57735
Nat.col	5	9	8	10	9	1
8µm	6	13	12	14	13	1
Mean					**10.38+/-1.38**	
	1	23	26	25	24.66667	1.527525
	2	18	21	24	21	3
10 ng/ml VEGF+ 75nM PI103	3	20	22	21	21	1
5hr	4	24	24	26	24.66667	1.154701
Nat.col	5	27	29	28	28	1
8µm	6	22	23	25	23.33333	1.527525
Mean					**23.77+/-2.64**	
	1	23	21	20	21.33333	1.527525
	2	23	25	26	24.66667	1.527525
10 ng/ml VEGF+ 125nM PI103	3	24	27	28	26.33333	2.081666
5hr	4	31	28	27	28.66667	2.081666
Nat.col	5	19	25	26	23.33333	3.785939
8µm	6	23	25	27	25	2
Mean					**24.88+/-2.5**	
	1	20	26	24	23.33333	3.05505
	2	22	24	26	24	2
10ng/ml VEGF+ 250nM PI103	3	30	25	28	27.66667	2.516611
5hr	4	22	20	21	21	1
Nat.col	5	24	27	25	25.33333	1.527525
8µm	6	27	27	28	27.33333	0.57735
Mean					**24.77+/-2.53**	
	1	61	65	63	63	2
	2	74	67	69	70	3.605551
10 ng/ml VEGF	3	62	64	60	62	2
5hr	4	73	65	69	69	4
Nat.col	5	59	63	65	62.33333	3.05505
8µm	6	64	68	62	64.66667	3.05505
Mean					**65.16+/-3.49**	

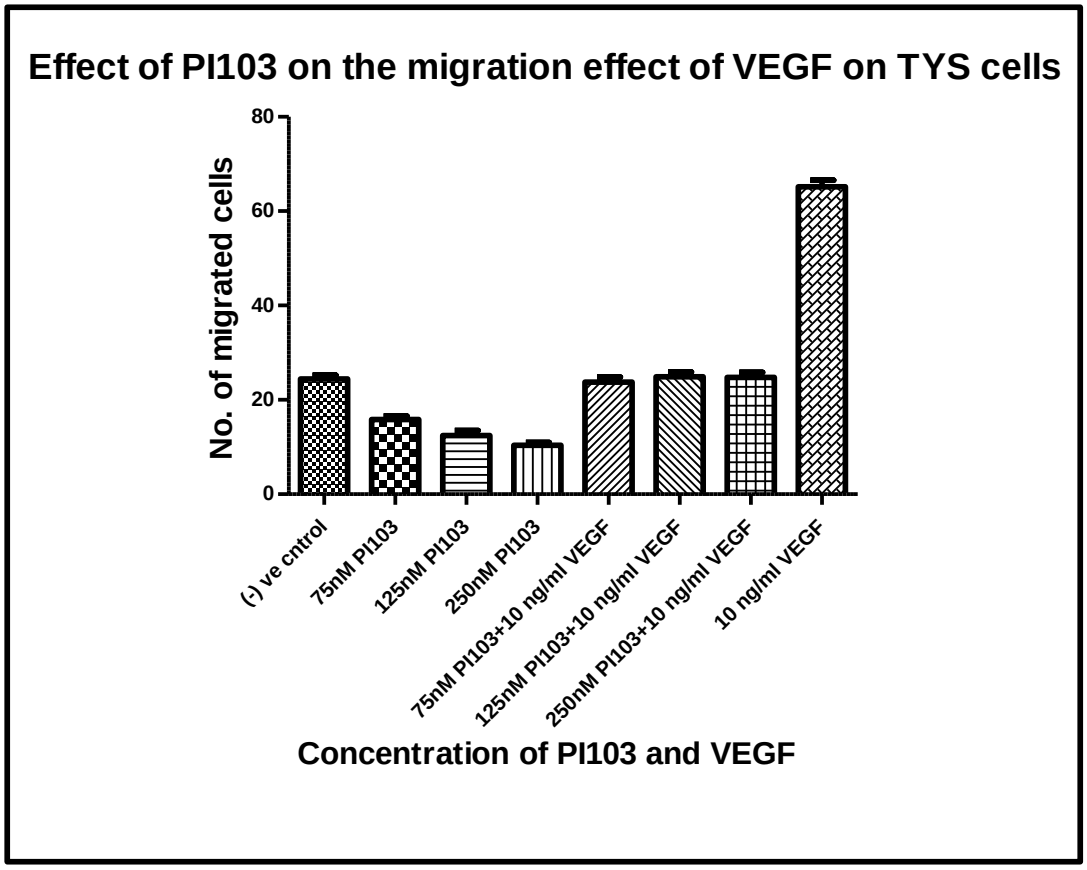
Summary of Data:

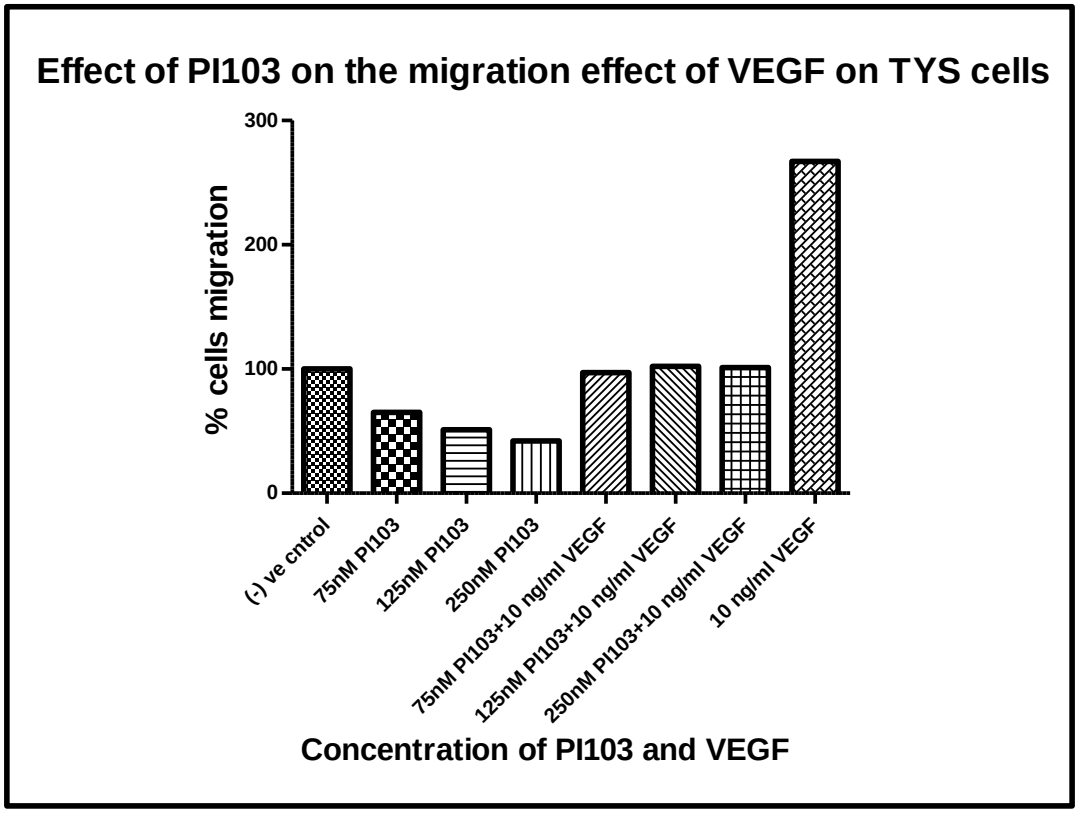
Chamber 1: HACAT with VEGF and PI103

5 hours incubation

8μm pore filter coated with native collagen
Base medium: SF-BSA (2 μg/ml)

	Mean	SD	% migration	Significance?	
				Bonferroni's (P<0.05)	Kruskal-Wallis & Dunnet's test (p<0.05)
SF-BSA	24.44	+/-1.85	100		
75nM PI103	15.83	+/- 1.73	65	Y	N
125nM PI103	12.44	+/-2.54	51	Y	N
250nM PI103	10.38	+/-1.38	42	Y	N
10 ng/ml VEGF+ 75nM PI103	23.77	+/-2.64	97	N	N
10 ng/ml VEGF+ 125nM PI103	24.88	+/-2.5	102	N	N
10 ng/ml VEGF+ 250nM PI103	24.77	+/-2.53	101	N	N
10 ng/ml VEGF	65.16	+/- 3.49	267	Y	N


Effect of PI103 on the migration effect of VEGF on TYS cells
No. of migrated cells
80
60
40
20
0
(-) ve cntrol
75nM PI103
125nM PI103
250nM PI103
75nM PI103+10 ng/ml VEGF
125nM PI103+10 ng/ml VEGF
250nM PI103+10 ng/ml VEGF
10 ng/ml VEGF
Concentration of PI103 and VEGF


Effect of PI103 on the migration effect of VEGF on TYS cells
% cells migration
300
200
100
0
(-) ve cntrol
75nM PI103
125nM PI103
250nM PI103
75nM PI103+10 ng/ml VEGF
125nM PI103+10 ng/ml VEGF
250nM PI103+10 ng/ml VEGF
10 ng/ml VEGF
Concentration of PI103 and VEGF

Appendix 12. Statistical Analysis: Boyden chamber Assay, TYS and VEGF

Parameter	
Table Analyzed	Data 1
One-way analysis of variance	
P value	< 0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	7
F	96.89
R square	0.9432
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	7.434
P value	0.2826
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	5642	6	940.3
Residual (within columns)	339.7	35	9.705
Total	5982	41	

Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
SF-BSA vs 1pg/ml VEGF121	-7.944	4.417	Yes	**	-13.84 to -2.053
SF-BSA vs 10pg/ml VEGF121	-17.61	9.791	Yes	***	-23.50 to -11.72
SF-BSA vs 100pg/ml VEGF121	-23.44	13.03	Yes	***	-29.34 to -17.55
SF-BSA vs 1ng/ml VEGF121	-29.06	16.15	Yes	***	-34.95 to -23.16
SF-BSA vs 10ng/ml VEGF121	-32.44	18.04	Yes	***	-38.34 to -26.55
SF-BSA vs 100ng/ml VEGF121	-4.944	2.749	No	ns	-10.84 to 0.9474
1pg/ml VEGF121 vs 10pg/ml VEGF121	-9.667	5.374	Yes	***	-15.56 to -3.775
1pg/ml VEGF121 vs 100pg/ml VEGF121	-15.50	8.618	Yes	***	-21.39 to -9.608
1pg/ml VEGF121 vs 1ng/ml VEGF121	-21.11	11.74	Yes	***	-27.00 to -15.22
1pg/ml VEGF121 vs 10ng/ml VEGF121	-24.50	13.62	Yes	***	-30.39 to -18.61
1pg/ml VEGF121 vs 100ng/ml VEGF121	3.000	1.668	No	ns	-2.892 to 8.892
10pg/ml VEGF121 vs 100pg/ml VEGF121	-5.833	3.243	No	ns	-11.73 to 0.05850
10pg/ml VEGF121 vs 1ng/ml VEGF121	-11.44	6.363	Yes	***	-17.34 to -5.553
10pg/ml VEGF121 vs 10ng/ml VEGF121	-14.83	8.247	Yes	***	-20.73 to -8.941
10pg/ml VEGF121 vs 100ng/ml VEGF121	12.67	7.042	Yes	***	6.775 to 18.56
100pg/ml VEGF121 vs 1ng/ml VEGF121	-5.611	3.120	No	ns	-11.50 to 0.2807
100pg/ml VEGF121 vs 10ng/ml VEGF121	-9.000	5.004	Yes	***	-14.89 to -3.108
100pg/ml VEGF121 vs 100ng/ml VEGF121	18.50	10.29	Yes	***	12.61 to 24.39
1ng/ml VEGF121 vs 10ng/ml VEGF121	-3.389	1.884	No	ns	-9.281 to 2.503
1ng/ml VEGF121 vs 100ng/ml VEGF121	24.11	13.41	Yes	***	18.22 to 30.00
10ng/ml VEGF121 vs 100ng/ml VEGF121	27.50	15.29	Yes	***	21.61 to 33.39

Appendix 13. Legends of the chemotaxis video clips

Video clip 4.1 Chemotaxis video of TR146 cells stimulated by VEGF.

Oral cancer cells (TR146) were stimulated to migrate towards VEGF in μ-slide chemotaxis chamber and this migration of TR146 cells towards the chemoattractant (VEGF) was statistically significant ($p<0.001$).

Video clip 4.2 Trajectory plot of TR146 cells migration towards VEGF.

Around 40 cells were randomly tracked and a trajectory plot was generated. The plot showed the migration of TR146 cells towards VEGF.

Video clip 4.3 Chemotaxis video of HaCaT cells treated with VEGF.

Chemotaxis video of normal keratinocyte cells showed no migration towards VEGF.

Video clip 4.4 Trajectory plot of HaCaT cell migration treated with VEGF.

A trajectory plot was also generated by randomly tracked HaCaT cells. No movement was observed among the tracked HaCaT cells.

Video clip 4.5 Chemotaxis video of TR146 cells treated with VEGF and PI103.

Oral cancer cells were not migrated towards VEGF and PI103. PI103 effectively blocked VEGF stimulated oral cancer cell migration.

Video clip 4.6 Trajectory plot of TR146 cells treated with VEGF and PI103.

Randomly selected TR146 cells were tracked and trajectory plot was created. Plot showed no migration of oral cancer cells towards VEGF and PI103.

Video clip 4.7 Chemotaxis video of TR146 cells treated with VEGF and LY294002.

TR146 cells showed no migration towards VEGF and LY294002, in contrast cells were migrated towards negative control (serum-free media). Statistical analysis showed directness of the migration was significant towards serum-free ($P<0.05$) but Cells were stayed inside the observation area of the chamber.

Video clip 4.8 Trajectory plot of TR146 cells treated with VEGF and LY294002.

Trajectory plot showed TR146 cells moved opposite to VEGF and LY294002 although a minimum distance.

Appendix 14. Optimizing of Akt antibodies (pAkt Ser473 and pAkt Thr 308) for IHC in Oral tumour samples

Plan of experiment

Primary antibodies
pAkt Thr 308: # 2965 (Cell signalling) (1:20, 1:50, 1:100)
pAkt Ser473: # 4060(Cell Signalling) (1:20, 1:50, 1:100)

Secondary antibodies
Goat anti-rabbit HRP conjugated (# 7074, Cell Signalling) (1:1000 and 1:3000)
Signal stain boost antibody detection reagent (#8114, Cell signaling)

Negative ctrl: No primary, Blocking peptide

Pre-treatment: Microwave with citrate buffer (pH 6), No Pre-treatment, Trypsin digestion

Diluent: 5%NGS-TBST, SS Antibody diluent (#8112, Cell signaling)

Staining: DAB in PBS

Results:

pAkt T308 Ab	2^0 Ab	Pre-treatment	Result
1:20	7074	Citrate, micro	No Staining
1:20	7074	No PT	No Staining
1:20	7074	Trypsin	No Staining
1:20	8114	Citrate, micro	No Staining
1:20	8114	No PT	No Staining
1:20	8114	Trypsin	No Staining
1:50	7074	Citrate, micro	No Staining
1:50	7074	No PT	No Staining
1:50	7074	Trypsin	No Staining
1:50	8114	Citrate, micro	Very good staining
1:50	8114	No PT	No Staining
1:50	8114	Trypsin	No Staining
1:100	7074	Citrate, micro	No Staining
1:100	7074	No PT	No Staining
1:100	7074	Trypsin	No Staining

1:100	8114	Citrate, micro	Very weak staining
1:100	8114	No PT	No Staining
1:100	8114	Trypsin	No Staining
pAkt S473 Ab	**2^0 Ab**	**Pre-treatment**	**Result**
1:20	7074	Citrate, micro	No Staining
1:20	7074	No PT	No Staining
1:20	7074	Trypsin	No Staining
1:20	8114	Citrate, micro	Very good Staining
1:20	8114	No PT	No Staining
1:20	8114	Trypsin	No Staining
1:50	7074	Citrate, micro	No Staining
1:50	7074	No PT	No Staining
1:50	7074	Trypsin	No Staining
1:50	8114	Citrate, micro	Weak staining
1:50	8114	No PT	No Staining
1:50	8114	Trypsin	No Staining
1:100	7074	Citrate, micro	No Staining
1:100	7074	No PT	No Staining
1:100	7074	Trypsin	No Staining
1:100	8114	Citrate, micro	No staining
1:100	8114	No PT	No Staining
1:100	8114	Trypsin	No Staining

Suggestions: 1:50 dilution for pAkt T308 and 1:20 dilution for pAkt S473 was selected for optimal IHC result. Signal boost detection reagent and Citrate buffer (microwave heating) were also selected for both the antibodies.

Appendix 15. HNSCC patient data with pAkt score

Pathology ID	Gender	Age	Site	Tobacco	Alcohol	Tumour Size	Nodal Status	Tumour grade	Recurrence	Follow-up (months)	Status	pAkt Thr308 score				pAkt Ser473 Score				HPV 16 status
												Obs 1	Obs 2	Obs 3	Avg score	Obs 1	Obs 2	Obs 3	Avg score	
01371006-1A	F	75	tongue	Y	N	T4	N2	3	N	4	D	9	6	7	7.3	3	2	1	2	Y
0187207-1HE	F	65	supraglottis	Y	H	T4	N0	2	N	31	A	7	8	5	6.5	1	1	0	0.6	N
01353006-1	F	63	retromolar trigone	Y	H	T4	N2	2	N	29	A	7	4	6	5.6	1	2	2	1.6	N
0059407-1	M	55	tonsil	Y	H	T3	N2	2	N	30	A	8	5	6	6.3	3	2	2	2.3	N
0109407-2F	F	59	tongue	Y	H	T2	N2	2	N	31	A	9	8	5	7.3	0	0	0	0	N
02338805-1	M	70	soft palate	Y	N	T1	N0	2	N	35	A	8	5	4	5.6	2	2	2	2	N
0338107-2G	M	75	tongue	Y	H	T2	N0	2	N	30	A	6	6	2	4.6	1	1	1	1	N
01864704-1B	F	78	retromolar trigone	Y	H	T2	N0	2	N	57	A	4	8	3	5	0	0	0	0	N
0164406-3C	M	65	tongue	Y	H	T4	N1	2	N	43	A	10	8	6	8	1	0	0	0.3	N
00148106-1	M	59	soft palate	Y	H	T2	N0	3	Y	34	D	7	5	8	6.5	2	3	2	2.3	N
01514806-3D	F	75	tongue	Y	N	T4	N2	3	N	4	D	8	5	7	6.5	0	0	0		N
01803103-1	M	67	floor of mouth	Y	M	T1	N0	1	N	60	A	7	4	3	4.5	2	1	1	1.3	N

0671203-1	M	69	oropharynx	Y	M	T4	N0	3	Y	44	D	6	8	4	6	0	0	0	0	N
0711302-1B	F	55	tongue	N	M	T2	N2	2	Y	60	A	4	4	4	4	3	3	1	2.3	N
01399006-1	F	59	alveolus	N	M	T4	N0	2	N	35	A	1	2	3	2	1	1	2	1.3	Y
02579406-1	F	59	tongue	Y	H	T2	N0	2	N	31	A	5	6	7	6	2	2	1	1.6	N
00232207-1	F	63	floor of mouth	Y	H	T1	N0	2	N	29	A	8	6	3	5.5	6	4	3	4.3	N
0507307-2B	F	63	floor of mouth	Y	H	T1	N0	2	N	29	A	5	8	4	5.5	0	0	0	0	N
012909199-2	M	64	tongue	Y	N	T1	N0	2	N	55	A	2	2	1	1.5	0	0	0	0	N
01020203-3K	M	62	tongue	N	H	T2	N2	2	Y	66	A	12	7	9	9.5	0	0	0	0	N
01258903-1C	M	64	floor of mouth	Y	H	T4	N0	2	Y	60	A	6	4	8	6	0	0	0	0	N
681/03-1B	M	74	retromolar trigone	Y	M	T1	N0	1	N	60	A	8	5	3	5.2	0	0	0	0	Y
00442204-1L	F	97	retromolar trigone	N	N	T2	N0	2	Y	8	D	3	2	1	2	1	0	0	0.3	Y
01642002-1A	M	60	tongue	Y	H	T2	N2	2	Y	40	A	6	7	3	5.4	0	0	0	0	N
00196904-1	F	97	retromolar trigone	N	N	T2	N2	2	Y	8	D	3	2	1	2	1	0	0	0.3	N
01404702-1B	M	60	tongue	Y	H	T2	N2	2	Y	40	A	5	7	6	6	1	1	1	0.9	N
0267305-3E	M	66	retromolar trigone	Y	H	T1	N0	2	N	60	A	1	2	3	2	0	0	0	0	N
0848002-3D	F	55	tongue	N	M	T2	N2	2	Y	60	A	6	8	3	5.6	0	0	0	0	N

60315505-1G	M	52	tongue	N	M	T1	N1	3	Y	54	A	8	7	5	6.5	0	0	0	0	N
00629005-1	F	46	tongue	Y	M	T1	N0	2	N	51	A	8	10	6	8	0	0	1	0.3	N
006/4/04-HED	F	63	soft palate	Y	M	T2	N2	3	N	60	A	1	3	2	2	1	1	1	1	N
01291204-1C	M	77	tongue	N	M	T1	N1	3	Y	37	D	6	7	7	6.6	0	1	1	0.6	N
0139006-1E	F	62	tongue	N	M	T1	N2	3	N	37	A	1	2	3	2	0	0	0	0	N
014235063H	F	59	alveolus	N	M	T2	N0	2	N	35	A	1	1	4	2	0	0	0	0	N
00642004-1	M	78	tongue	Y	H	T2	N1	2	N	60	A	8	7	8	7.5	1	1	1	0.9	N
0044505-1	M	52	tongue	N	M	T1	N1	3	Y	54	A	8	4	5	5.5	1	1	2	1.3	N
01076404-1B	M	67	floor of mouth	Y	M	T1	N0	1	N	60	A	1	3	2	1.9	0	0	0	0	N
021814105-2L	M	62	tongue	N	M	T1	N0	2	N	45	A	6	3	3	4	0	0	0	0	Y
01218406-1	F	62	tongue	N	M	T1	N2	3	N	37	A	7	5	5	5.5	1	0	0	0.3	N
00624606-1	M	62	tongue	N	M	T4	N0	3	N	41	A	4	3	1	2.6	1	2	1	1.3	N
01399406-4F	F	81	floor of mouth	Y	H	T1	N1	3	N	37	A	8	5	7	6.5	1	1	0	0.6	Y
0958906-1C	M	67	tongue	Y	H	T2	N1	2	N	39	A	7	7	6	6.5	0	0	0	0	N
01298705-1	M	84	alveolus	Y	M	T4	N0	2	Y	7	D	7	6	6	6.5	2	1	1	1.3	N
0012706-1	M	65	tongue	Y	H	T4	N1	2	N	43	A	8	5	7	6.5	0	0	0	0	Y
01292806-2K	M	69	retromolar trigone	Y	M	T4	N2	3	Y	38	A	9	5	7	7	0	0	0	0	N
01596705	F	78	buccal	N	M	T2	N0	2	Y	17	D	7	4	4	5	1	1	1	1	N

-1			mucosa																
40727066-1	M	67	tongue	Y	H	T2	N1	2	N	39	A	8	8	6	7.3	0	0	1	0.3	N
00965006-3C	F	66	floor of mouth	Y	N	T2	N2	3	N	39	A	8	7	4	6.3	0	0	1	0.3	N
02318405-1	M	62	soft palate	Y	H	T2	N2	2	N	42	A	4	9	8	7	1	1	0	0.6	N
02060804-1	F	66	alveolus	N	H	T1	N2	2	Y	54	A	6	6	7	6.3	1	0	1	0.6	N
01029003-1	M	64	floor of mouth	Y	H	T4	N0	2	Y	60	D	6	5	4	5	1	1	1	1	N
0055407-1	M	75	tongue	Y	H	T2	N0	2	N	30	A	7	8	6	7	1	1	2	1.3	N
1993205-1	M	84	soft palate	Y	M	T4	N0	2	Y	30	D	8	6	10	8	4	2	2	2.6	N
0213305-1B	F	62	floor of mouth	Y	H	T1	N0	2	N	54	A	8	7	5	6.6	0	0	0	0	N
0202204-1	F	62	floor of mouth	Y	H	T1	N0	2	N	54	A	2	2	2	2	1	1	1	1	N
1960104-1	M	66	retromolar trigone	Y	H	T1	N0	2	N	60	A	9	4	4	5.6	0	0	0	0	N
0279203-1	M	36	tongue	Y	H	T1	N0	1	N	41	A	6	6	6	6	1	1	1	1	N
00486606-1A	M	36	tongue	Y	H	T1	N0	1	N	41	A	4	7	6	5.6	0	0	0	0	N

Tobacco: Y-Yes, N-No; Alcohol: N-None drinker, M-Medium drinker and H-Heavy drinker; Status: D-Dead, A-Alive

Appendix 16. Statistical analysis of pAkt IHC score and HNSCC patient data

General Linear Model: Multivariate

Between-Subjects Factors

		Value Label	N
Smoking	0	non-smoker	16
	1	Smoker	42
Alcohol	0	Nondrinker	7
	1	meudim drinker	21
	2	heavy drinker	30
Nodalstatusgrp	0	No lymph node metas	31
	1	Lymp node metas 1&2	27
agegrp	0	<65	30
	1	>=65	28

Tests of Between-Subjects Effects

Dependent Variable: pAkt T308

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	138.717[a]	18	7.706	4.059	.000
Intercept	518.039	1	518.039	272.876	.000
Smoking	10.063	1	10.063	5.301	.027
Alcohol	40.076	2	20.038	10.555	.000
Nodalstatusgrp	2.479	1	2.479	1.306	.260
agegrp	9.460	1	9.460	4.983	.031
Smoking * Alcohol	12.144	2	6.072	3.198	.052
Smoking * Nodalstatusgrp	7.815	1	7.815	4.116	.049
Smoking * agegrp	.020	1	.020	.011	.919
Alcohol * Nodalstatusgrp	13.506	2	6.753	3.557	.038
Alcohol * agegrp	20.155	2	10.077	5.308	.009
Nodalstatusgrp * agegrp	5.317	1	5.317	2.801	.102
Smoking * Alcohol * Nodalstatusgrp	.323	1	.323	.170	.682
Smoking * Alcohol * agegrp	.158	1	.158	.083	.774
Smoking * Nodalstatusgrp * agegrp	11.391	1	11.391	6.000	.019
Alcohol * Nodalstatusgrp * agegrp	10.874	1	10.874	5.728	.022
Smoking * Alcohol * Nodalstatusgrp * agegrp	.000	0	.	.	.
Error	74.039	39	1.898		
Total	1894.330	58			
Corrected Total	212.756	57			

a. R Squared = .652 (Adjusted R Squared = .491)

Cox Regression (Adjusted)

Categorical Variable Codings[c,d,e,f,g]

		Frequency	(1)[b]	(2)
Alcohol[a]	0=Nondrinker	7	1	0
	1=meudim drinker	21	0	1
	2=heavy drinker	30	0	0
Tumoursizegrp[a]	0=T1-T2	43	1	
	1=T3-T4	15	0	
pAkts473grp[a]	0=No phosphorylation	23	1	0
	1=Low phosphorylation	30	0	1
	2=Medium phosphorylation	5	0	0
agegrp[a]	0=<65	30	1	
	1=>=65	28	0	
HPV[a]	0=negative	29	1	
	1=positive	29	0	

a. Indicator Parameter Coding

b. The (0,1) variable has been recoded, so its coefficients will not be the same as for indicator (0,1) coding.

c. Category variable: Alcohol

d. Category variable: Tumoursizegrp

e. Category variable: pAkts473grp

f. Category variable: agegrp

g. Category variable: HPV

Block 1: Method = Backward Stepwise (Likelihood Ratio)

Omnibus Tests of Model Coefficients[b]

		Overall (score)			Change From Previous Step			Change From Previous Block		
Step	-2 Log Likelihood	Chi-square	df	Sig.	Chi-square	df	Sig.	Chi-square	df	Sig.
1[a]	37.254	37.016	7	.000	44.694	7	.000	44.694	7	.000

a. Variable(s) Entered at Step Number 1: HPV Tumoursizegrp agegrp pAkts473grp Alcohol

b. Beginning Block Number 1. Method = Backward Stepwise (Likelihood Ratio)

Variables in the Equation

								95.0% CI for Exp(B)	
		B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1	HPV	-4.493	1.578	8.103	1	.004	.011	.001	.247
	Tumoursizegrp	-2.724	.970	7.895	1	.005	.066	.010	.439
	agegrp	-3.847	1.436	7.176	1	.007	.021	.001	.356
	pAkts473grp			10.667	2	.005			
	pAkts473grp(1)	-6.081	1.883	10.426	1	.001	.002	.000	.092
	pAkts473grp(2)	-3.068	1.461	4.410	1	.036	.047	.003	.815
	Alcohol			7.763	2	.021			
	Alcohol(1)	3.900	1.422	7.523	1	.006	49.391	3.044	801.498
	Alcohol(2)	.990	1.044	.900	1	.343	2.692	.348	20.829

Appendix 17. Published papers

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Is there a pAkt between VEGF and oral cancer cell migration?

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ABSTRACT

The PI3K-Akt signalling pathway is a well-established driver of cancer progression. One key process promoted by Akt phosphorylation is tumour cell motility; however the mechanism of VEGF-induced Akt phosphorylation leading to motility remains poorly understood. Previously, we have shown that Akt phosphorylation induced by different factors causes both stimulation and inhibition of motility in different cell types. However, differential phosphorylation of Akt at T308 and S473 residues by VEGF and its role in head and neck cancer cell motility and progression is unknown. The cell lines investigated in this study exhibited a change in phosphorylation of Akt in response to VEGF. However, in terms of motility, VEGF stimulated oral cancer and its associated cell lines, but not normal keratinocytes or oral mucosal fibroblasts. The addition of a PI3 kinase and mTOR inhibitor, inhibited the phosphorylation of Akt and also effectively blocked VEGF-induced oral cancer cell motility, whereas only the PI3 kinase inhibitor blocked oral cancer associated fibroblast cell motility. This study therefore discloses that two different mechanisms of Akt phosphorylation control the motility potential of different cell lines. Akt phosphorylated at both residues controls oral cancer cell motility. Furthermore, immunohistochemical analysis of VEGF positive human head and neck tumour tissues showed a significant increase in Akt phosphorylation at the T308 residue, suggesting that pAkt T308 may be associated with tumour progression *in vivo*.

1. Introduction

Cell motility or migration is an essential part of most tumour pathways. Cells need to migrate away from their microenvironment to enable the tumour to spread or metastasise. Growth factors and matrix macromolecules are essential for the movement of cells [1]. Such movement requires a reorganisation of the actin cytoskeleton, which is under the control of many pathways including the PI3 kinase signal transduction pathway. The Class 1 PI3 kinases are a set of lipid kinases that phosphorylate the relatively abundant membrane phospholipid, phosphatidylinositol 4, 5 biphosphate (PIP2), generating small quantities of phosphatidylinositol 3, 4, 5 triphosphate (PIP3). This latter lipid signal controls a diverse set of effector molecules including the Akt group of oncogenic kinases (also known as protein kinase B) [2]. Activation of Akt, a 60 kDa serine/threonine kinase, depends on PI3K [3]. Increase of cellular PIP3 by PI3K eventually allows the activation of Akt by phosphorylation at residues T308 and S473 [4]. This activation is completed by structural modification stimulated by PI3K-dependent kinase-1 (PDK-1)-dependent phosphorylation at T308 and stabilisation by mTORC2-dependent phosphorylation at S473 [5].

We have previously shown that the PI3 kinase and Akt pathways are essential for the migration of fibroblasts in response to added factors such as Epidermal growth factor (EGF) and Transforming growth factor alpha (TGFα) [6]. The addition of PI3 kinase inhibitors blocks the migration stimulating activity of EGF and TGFα, the data indicating that both growth factors increase phosphorylation of Akt. Inhibition of PI3K activity blocks migration stimulated by G protein-coupled receptors or by receptor tyrosine kinases, signifying that PI3K has a vital function in cell migration [7]. Higher expression of phosphorylated Akt has also been reported in oral cancer, in comparison to normal mucosa and pre-cancerous tissue [8]. Vascular endothelial growth factor (VEGF) has been reported to stimulate the proliferation of endothelial cells and to enhance vascular permeability and survival [9]. In addition, it has also been shown that VEGF positivity, as assessed (or identified) by immunohistochemistry, is a functional indicator of poor prognosis in oral cancer.

VEGF status may become a significant prognostic factor in head and neck cancer [10,11]. Over-expressed VEGF acts as an effective angiogenic cytokine, stimulating endothelial cells thus promoting angiogenesis in solid tumours such as breast or ovarian carcinomas [12]. VEGF is a disulphide-linked dimeric glycoprotein which has six isoforms generated by alternative splicing, typically the 121 and 165 isoforms are the most common. These VEGF isoforms vary in their heparin binding capacity, in addition to their ability to bind the tyrosine-kinase receptors

Abbreviations: pAkt T308, phosphorylated Akt at threonine 308; pAkt S473, phosphorylated Akt at serine 473; VEGF, vascular endothelial growth factor; PI3K, phosphatidylinositide 3-kinases; HNSCC, head & neck squamous cell carcinoma; OSCC, oral squamous cell carcinoma.

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VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1) and to neuropilin-1 and neuropilin-2 [13]. $VEGF_{121}$ is considered to be more angiogenic and tumourigenic than the other isoforms [14].

Oral cancer, a malignant neoplasm that affects the tissues of the mouth, is the eighth most common cause of cancer-related deaths worldwide [15,16]. Globally, more than 90% of these malignancies are squamous cell carcinomas (SCC) occurring in the mucous membranes and oropharynx [17]. There were some 399,546 new cases of oral cancer and other pharyngeal cancers according to the GLOBOCAN 2008 database, collated by the International Agency for Research on Cancer [18]. This incidence is thought to be due to increased use of alcohol and tobacco. Oral cancer still has a poor survival rate, with a high occurrence of metastases, even though there has been significant progress in cancer treatment over the past few decades [19].

In this study, we aimed to establish the role of the PI3K-Akt pathway in $VEGF_{121}$ induced migration of oral cancer cells. The resultant data will help us to extend the spectrum of known biological activities of this pathway and to propose that inhibition of this pathway will be a suitable target for chemotherapeutic drug design to control oral cancer cell metastasis.

2. Materials and methods

2.1. Reagents, antibodies and inhibitors

The primary antibodies used were: rabbit monoclonal anti-pAkt T308 (# 2965), anti-pAkt S473 (# 4060), anti-pan Akt (# 4691) (all Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit polyclonal anti-VEGF A(A-20) (# sc152, Santa Cruz Biotechnology, TX, USA) and mouse monoclonal anti-GAPDH (# MAB374, Millipore, Darmstadt, Germany). The secondary antibodies used were goat anti-rabbit HRP conjugated (# 7074, Cell Signaling Technology), rabbit anti-mouse HRP conjugated (# P0260, Dako, Cambridgeshire, UK) and biotinylated anti-rabbit (BA-2020, Vector Laboratories, CA, USA). Recombinant Human $VEGF_{121}$ (# 10–1296) was purchased from Insight Biotechnology Ltd., Middlesex, UK. The PI3K-Akt pathway inhibitors LY294002 (# 9901) and PI103 (# 528100) were purchased from Merck Calbiochem, Darmstadt, Germany. The blocking peptides for pAkt S473 Ab (#1140) and pAkt T308 Ab (#1145B) were also purchased from Cell Signaling technology, Inc.

2.2. Cell culture

The highly differentiated oral mucosal squamous cell carcinoma cell line (TR-146) originated from cheek mucosa and was derived from lymph node. The stromal line (PM1) originated from forehead skin and was derived from dysplastic lesion. These lines were a kind gift from Dr. Dorothy Couch, Dundee Dental School. The oral adenoid squamous cell carcinoma (OASCC) cell line (TYS), derived from a minor salivary gland was a kind gift from Dr. Koji Harada, University of Tokushima, Japan. Normal adult keratinocytes (HaCaT) and normal oral mucosal fibroblasts (MM1) were a kind gift from Prof S.L. Schor and Dr. M. Macluskey, Dundee Dental School, UK, respectively. Mouth cancer-associated fibroblast cell line, COM D25 was isolated in-house from explant culture of a biopsy from the Oral Surgery Clinic, Ninewells Hospital, Dundee. All the cells were cultured at 37 °C and 5% CO_2 in MEM media supplemented with 10% (v/v) foetal calf serum (FCS) and 200 mM glutamine.

2.3. SDS-PAGE and Western blotting

Cells grown on 60 mm culture dishes were lysed on ice with RIPA buffer (50 mM Tris HCl, 150 mM NaCl, pH 7.4; 0.1% w/v SDS, 1% v/v Triton x-100, 1% w/v sodium deoxycholate and 5 mM EDTA) containing protease inhibitors (Roche Applied Science, IN, USA). RIPA buffer with added phosphatase inhibitors (Roche Applied Science, IN, USA) was used to lyse the cells treated with different concentration of VEGF. Lysates were clarified by centrifugation at 13,000 rpm for 5 min. Samples were then mixed with an equal volume of Laemmli sample loading buffer (BioRAD, CA, USA) including 5% (v/v 2-mercaptoethanol). Samples were heated at 95 °C for 5 min and loaded onto 'Any kD' SDS-PAGE BioRad TGX precast gels. After completion of SDS PAGE, proteins were electro-transferred onto nitrocellulose transfer membrane (0.45 μm, Whatman, Buckinghamshire, UK) and then immunoblotted with anti-pAkt T308 (1:1000), anti-pAkt S473 (1:2000) , anti-pan Akt (1:1000), anti-GAPDH (1:500), goat anti-rabbit HRP conjugated secondary antibody (1:2000) and rabbit anti-mouse HRP conjugated secondary antibody (1: 10,000). Immunoblots were developed using Immun-Star WesternC Kit (BioRad). Loading was controlled against GAPDH expression.

2.4. Boyden chamber migration assay

A 48-well Boyden chamber (Neuroprobe, Inc., MD, USA) was used for the *in vitro* migration assays as previously described [20]. In brief, cells suspended in serum-free MEM with bovine serum albumin (2 μg/ml) (SF-BSA) were seeded into the upper compartment of the chamber. The lower compartment was filled with different concentrations of $VEGF_{121}$ and inhibitors, diluted with SF-BSA. The two compartments were separated by a porous membrane filter (8 μm, Costar, UK) coated with type 1 native collagen. The chambers were incubated for 5 h at 37 °C. The filter was then washed twice in PBS, fixed in cold methanol and stained either with Mayer's (#MHS 32, Sigma-Aldrich, MO, USA) or Gills 3 (#095903, Brunel Microscope, Wiltshire, UK) haematoxylin overnight. The cells on the upper surface of the filter were scraped off with a cotton swab. The membrane was then mounted onto a glass slide and examined under bright field illumination at a magnification of ×200. Six replicate wells were used per variable. The numbers of migrated cells adherent to the lower surface of the membrane were counted in 3 random fields per well *i.e.* 18 fields per variable. Data were expressed as mean cell number per field ± SEM. When comparing different variables, results were expressed as a percentage of the controls.

2.5. Collagen gel migration assay

The collagen gel migration assay was performed as previously described [2]. Type I collagen from rat tail tendons was used to make 2 ml collagen gels in 35 mm plastic tissue culture dishes as described earlier [21]. Collagen gels were overlaid with 1 ml of either serum-free MEM (SF-MEM) or SF-MEM containing 4× the final concentration of $VEGF_{121}$. Confluent stock cultures of cells were then harvested, resuspended in growth medium containing 4% (v/v) FCS at the desired concentration and 1 ml aliquots were added to the overlaid gels. Considering the 2 ml volume of gel, 1 ml medium overlay and 1 ml cell inoculum, this procedure gives a final concentration of 1% (v/v) serum in both control and test cultures. Cells attached to the surface of the gel within 1 h and started to migrate into the underlying 3D gel within 24 h. Four days after plating, the number of cells remaining on the surface or that had migrated into the gel were determined by microscopic observation of 10 randomly selected fields in each of the duplicate cultures. Cell migration was expressed by the number of cells that migrated into the 3D gel, as a percentage of the total number of cells present (Mean ± s.e.m). When comparing different variables, results were expressed as a percentage of the controls.

2.6. Immunohistochemistry

64 HNSCC, 22 dysplastic and 11 normal oral mucosal tissue samples were collected by Dr. M. Macluskey, after ethical approval was granted, at Ninewells Hospital (Dundee) and the Royal Infirmary (Aberdeen) and were stored at Tayside tissue bank.

The paraffin-embedded HNSCC and dysplastic tissues were cut into 5-μm sections, dewaxed in xylene and then rehydrated in serial ethanol solutions, before washing in distilled water for 5 min. Endogenous peroxidase activity was blocked by incubation of the sections in 3% (v/v) hydrogen peroxide in phosphate buffered saline (PBS) for 10 min. The paraffin-embedded specimens were pre-treated with 0.1% (w/v) protease XXIV (Sigma-Aldrich) in PBS at room temperature for 30 min. Sections were incubated in normal goat serum (NGS) (Diagnostics, Scotland) at room temperature for 20 min prior to incubation with VEGF A antibody (1:100) at 4 °C overnight. The sections were then incubated with the biotinylated anti-rabbit secondary antibody followed by the avidin–biotin complex (Elite Vectastain Reagent Kit, Vector Labs). Visualisation was achieved by incubation with 3, 3′-diaminobenzidine (DAB) (Sigma-Aldrich) for 5 min and counterstaining with Mayer's haematoxylin and eosin. Sections were then dehydrated in graded alcohols and mounted in a xylene-based solvent, DPX (Merck).

VEGF A positive HNSCC samples were then collected and probed for pAkt T308, pAkt S473 and pan Akt according to the manufacturer's instructions. In brief, after the deparaffinisation and rehydration processes, antigens were unmasked by boiling in 10 mM sodium citrate (pH 6.0) buffer using a microwave, followed by maintenance at a sub-boiling temperature for 10 min and then cooling for 30 min on the bench top. 3% (v/v) H_2O_2 was then used as a peroxidase blocker and TBST (Tris buffered saline with 0.1% Tween 20) for washing. Sections were then blocked with 5% (v/v) normal goat serum plus TBST for 1 h at room temperature. Sections were then incubated with antibodies against pAkt S473 (1: 50), pAkt T308 (1:50) and pan Akt (1:300) diluted in 5% (v/v) NGS/TBST in a humidified chamber overnight at 4 °C. After equilibration, sections were then washed three times with TBST and then incubated in signal stain boost detection reagent (HRP, rabbit # 8114, Cell Signaling Technology, Inc.) for 30 min at room temperature. Visualisation, rehydration and mounting processes were then followed as described above. Normal oral mucosal tissues were used as negative control. Blocking the pAkt antibodies by the respective blocking peptide was done by adding twice the volume of peptide as volume of antibody used, in a total of 100 μl. These tissues were also used as negative control. Three independent observers scored the tissues as % area stained multiplied by intensity of staining.

2.7. Statistical analysis

The data was analysed using the statistical package IBM SPSS 19.0. Comparisons between the tissues were carried out using a Mann–Whitney *U* test with a 95% confidence interval. Differences in cell migration were analysed by Kruskal–Wallis test and Bonferroni with Dunn's post-test. Differences were considered significant when the p value was less than 0.05.

3. Results

3.1. Phosphorylation of Akt is VEGF concentration, cell line and time dependent

Phosphorylation of Akt at S473 was increased in OASCC (TYS), normal oral fibroblasts (MM1), cells from a dysplastic lesion (PM1) and OSCC (TR 146) and was fairly constant in normal keratinocytes (HaCaT) and mouth cancer-associated fibroblasts (COM D25) with increasing VEGF concentration (Fig. 1A). The TYS, HaCaT, COM D25 and PM1 exhibited a linear decrease and MM1 and TR 146 cells displayed an increase in Akt phosphorylation at T308 (Fig. 1B). Expression of total Akt was not upregulated in HaCaT and TYS cells by addition of exogenous VEGF (Fig. 1C). Phosphorylation of Akt, both at T308 and S473, can be blocked by the PI3K-Akt pathway inhibitors, LY294002 except for TR146 cells in which S473 was not inhibited (Fig. 1D) whereas PI103 blocked Akt phosphorylation at both residues (Fig. 1E). To observe the effect of time on the phosphorylation of Akt, the cells were incubated with VEGF for 15 min, 5 h and 24 h. Activation of pAkt at residues T308 and S473 was fairly constant in response to VEGF for each time period for TYS cells but gradually increased for TR146 cells, whereas there was either very low or no activation of Akt after 24 h for HaCaT, MM1 and PM1 (Fig. 1F).

3.2. VEGF can stimulate oral adeno-squamous cancer and cancer-associated fibroblast cell migration and can be blocked by LY294002 and PI103

Cell migration experiments using a Modified Boyden chamber assay were performed. Different concentrations of VEGF were used to investigate the role of this growth factor in the migration of normal adult keratinocytes (HaCaT), normal oral mucosal fibroblasts (MM1), mouth cancer-associated fibroblast (COM D25) and OASCC (TYS). HaCaT and MM1cells were not stimulated to migrate in response to VEGF (Fig. 2A, B). However, VEGF stimulated the migration of TYS and COM D25 cells (Fig. 2C, D) and this migration displayed a dose response effect with maximal stimulation at approximately 10 ng/ml VEGF ($p < 0.05$) (figure not shown). A cell permeable, potent, reversible and specific PI3K inhibitor, LY294002, which acts on the ATP binding site of the enzyme, had no effect on the migration of these cells either alone or in combination with VEGF (Fig. 2C, D). A blocking effect of LY294002 was observed at concentrations between 1 μM and 6 μM. No effect on the migration of MM1 and HaCaT was observed in response to LY294002 alone or in combination with VEGF (Fig. 2A, B). PI103, another potent, cell-permeable, ATP-competitive PI3K and mTORC1/2 inhibitor was added at 75 nM–250 nM and its effect on cell migration was observed. PI103 reduced HaCaT, MM1 and TYS cell migration from baseline to below the baseline level ($p < 0.05$) and showed no effect in combination with VEGF (Fig. 2A, B, C). PI103 alone had no effect and stimulated COM D25 cell migration in combination with VEGF (Fig. 2D).

3.3. VEGF can stimulate oral cancer cells and cells from a dysplastic lesion to migrate and can be blocked by PI103

Some of the cells lines investigated here did not migrate in the Modified Boyden chamber assay and therefore, a 3D collagen gel migration assay developed in other studies was used. After initial experiments to determine suitable concentrations of VEGF and the Akt inhibitors, the following concentrations were used in the collagen gel assay: 10 ng/ml $VEGF_{121}$, 6 μM LY294002 and 125 nM PI103. Normal oral fibroblasts (MM1) were not stimulated to migrate in response to VEGF (Fig. 3A), whereas VEGF stimulated the migration of cancer-associated fibroblasts (COM D25) (Fig. 3B, $p < 0.05$), cells from a dysplastic lesion (PM1) (Fig. 3C, $p < 0.05$) and OSCC (TR146) (Fig. 3D, $p < 0.05$) after 4 days of treatment. LY294002 and PI103 alone and in combination with VEGF had no effect on the MM1 and COM D25 cell migration (Fig. 3A, B). These two inhibitors alone also had no effect on the migration of PM1 (Fig. 3C) and TR 146 cells (Fig. 3D). LY294002, not PI103, in combination with VEGF stimulated the migration of these cells ($p < 0.05$).

3.4. Akt T308 phosphorylation is higher in the VEGF positive carcinoma compared to that of Akt S473

Tissues were stained with VEGF A antibody by immunohistochemistry and VEGF A expression was significantly elevated in HNSCC patients compared to dysplastic patients (Fig. 4A) ($p = 0.001$). VEGF positive carcinoma patient samples were then stained with pAkt S473, pAkt T308 and pan Akt antibodies. Some samples which were highly stained for pAkt S473 and pAkt T308 were then selected and tested with the blocking peptide for the respective antibody and were used as negative controls. No staining was observed in the antibody plus blocking peptide treated tissues and this confirms the specificity of the antibody. Normal oral mucosal tissue samples were also treated with pAkt antibodies and no staining was observed except with pan Akt (Fig. 4B). Probing for pAkt activated at

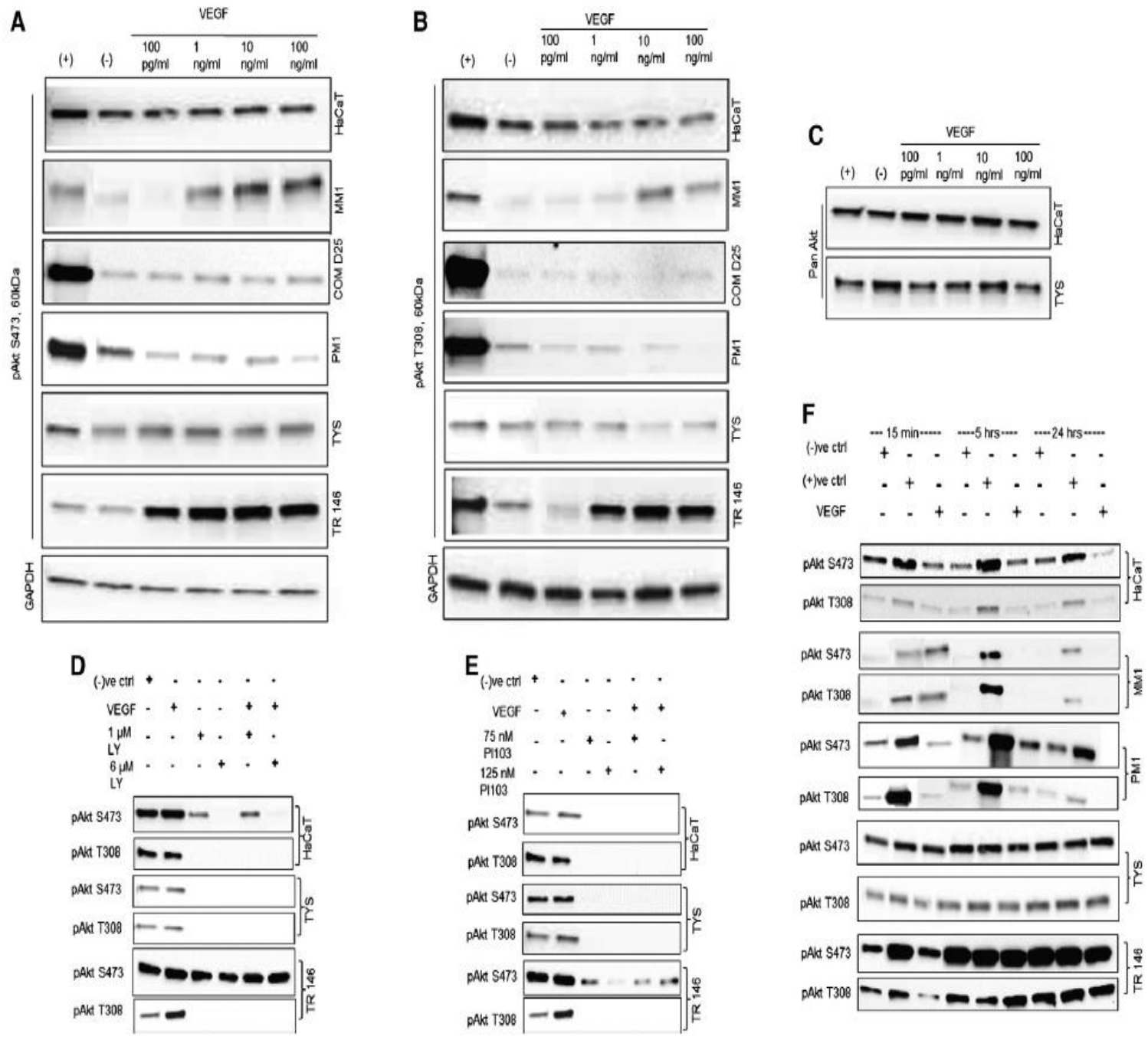

Fig. 1. Western blot experiments for Akt phosphorylation in 6 different cell lines with a spectrum of VEGF concentrations and inhibitors. (A) pAkt S473 phosphorylation in normal keratinocytes (HaCaT), normal oral fibroblasts (MM1), mouth cancer-associated fibroblasts (COM D25), cells from a dysplastic lesion (PM1), OASCC (TYS) and OSCC (TR 146) with VEGF concentrations of 100 pg/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml. All the cells were treated with VEGF for 15 min. (B) pAkt T308 phosphorylation in the same cell lines with the same VEGF concentrations as (A). Variable Akt phosphorylation at T308 was observed with increasing VEGF concentration in different cell lines. GAPDH was used as a loading control. (C) HaCaT and TYS cells showed a fairly constant presence of total Akt with a range of increasing VEGF concentrations. (D) Cells treated with LY294002 for 15 min blocked the phosphorylation of Akt at both residues except for TR146. S473 phosphorylation was not inhibited by LY294002 in the TR146 cells. (E) Phosphorylation of Akt at T308 and S473 residues was blocked by PI103 in HaCaT, TYS and TR146 cells. (F) VEGF treatment of TYS cells for various times caused a fairly constant level of Akt phosphorylation at both S473 and T308 but gradually increased for TR146. By contrast, after 24 h of VEGF exposure either very low or no phosphorylation of Akt at residues T308 and S473 was observed in HaCaT, MM1 and PM1 cells. All the blots were cropped from the original images. (+) denotes the positive control (FCS media) and (−) denotes the negative control (serum free media).

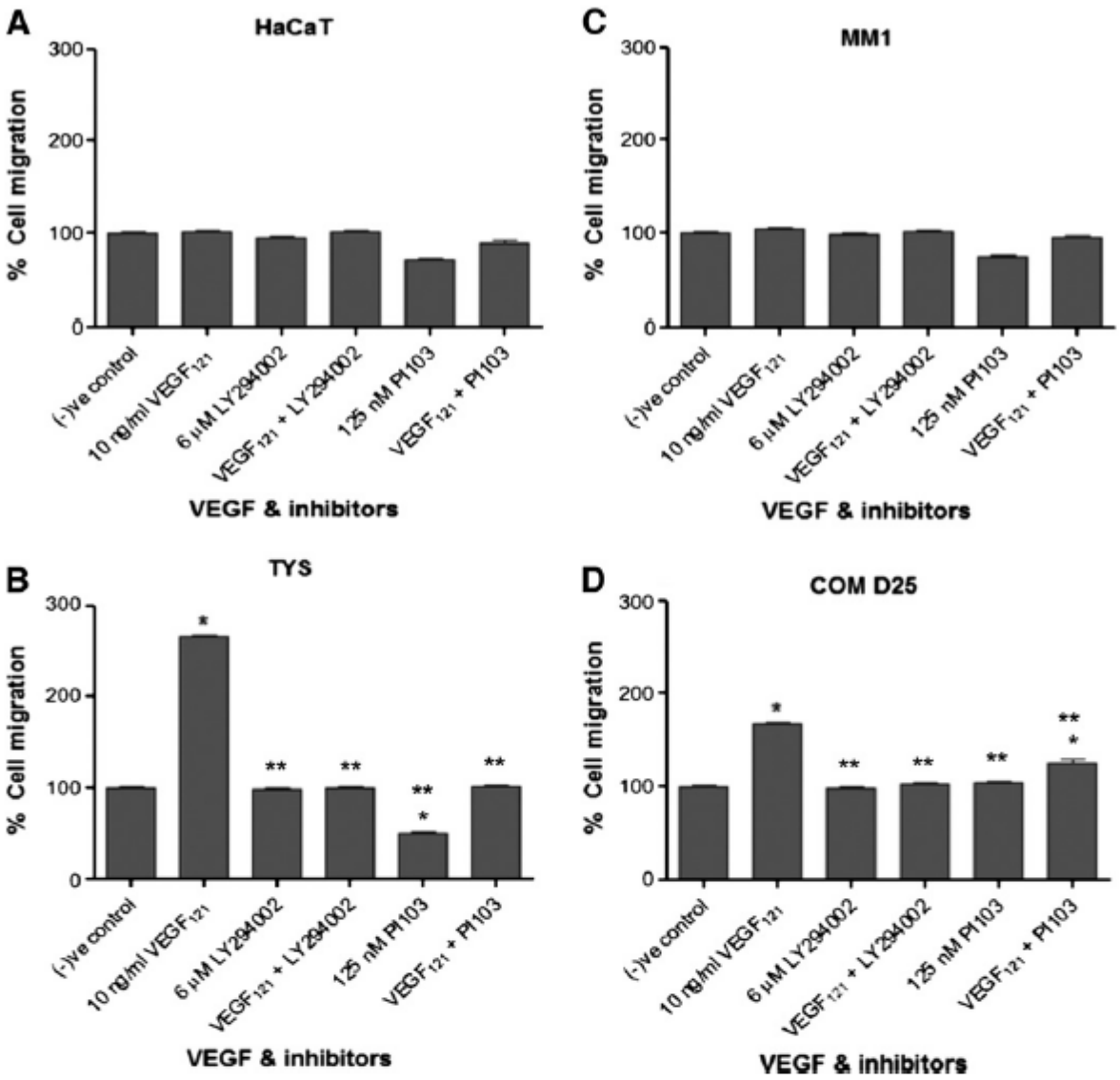

Fig. 2. Variable migratory response of different cell lines treated with VEGF and PI3K-Akt pathway inhibitors obtained by Boyden chamber migration assay compared with negative control. (A) Normal keratinocytes (HaCaT) ($n = 35$) and (B) Oral mucosal fibroblasts (MM1) ($n = 40$) were not stimulated to migrate by VEGF (baseline, $n = 32$ & 38, respectively) ($p > 0.05$). LY294002 had no effect either alone or in combination with VEGF on the migration of HaCaT ($n = 30$ & 33, respectively) and MM1 cells ($n = 37$ & 38, respectively). PI103 alone inhibits the migration of HaCaT ($n = 10$) and MM1 ($n = 21$) to below the baseline (baseline, $n = 14$ & 21, respectively) and has no effect in combination with VEGF ($n = 13$ & 27, respectively). (C) OASCC (TYS) ($n = 90$) and (D) Mouth cancer-associated fibroblast (COM D25) ($n = 50$) were stimulated to migrate through the filters by VEGF (baseline, $n = 34$ & 30, respectively) ($p < 0.05$). There is no effect of LY294002 alone or in combination with VEGF on TYS ($n = 35$ & 34, respectively) and COM D25 ($n = 29$ & 32, respectively) on cell migration. PI103 alone reduced TYS ($n = 13$) cell migration from baseline to below the baseline (baseline, $n = 24$) and showed no effect in combination with VEGF ($n = 25$). However, PI103 had no effect alone on COM D25 cell migration ($n = 26$) and stimulated migration ($n = 31$) ($p < 0.05$) in combination with VEGF (baseline, $n = 25$). n = number of cells migrated. Asterisk (*) indicates the significant changes compared with negative control. Double asterisk (**) indicates the significant changes compared with VEGF.

either residue S473 or T308 revealed higher staining in HNSCC tissues compared to normal tissues ($p < 0.05$) (Fig. 4C). In HNSCC patient samples, higher phosphorylation of pAkt T308 was observed, which was highly significant compared to that of pAkt S473 ($p < 0.001$) (Fig. 4D). Keratinocytes, fibroblasts, inflammatory infiltrates, blood vessels and keratin pearls produced strong pAkt T308 staining in the carcinoma samples, especially in the nucleus of the tumour keratinocytes (Figure not shown).

4. Discussion

We have investigated the relationship between VEGF treatment, Akt activity and cell migration in human cell lines. In summary, we have tested six different cell lines in this study to represent the stages of tumour progression ranging from normal to metastatic cells. In all of the lines tested, VEGF had an effect on phosphorylation of Akt (Fig. 1). The response of some of the cell lines to addition of exogenous VEGF, was an increase in phosphorylation of S473 (TR146, TYS, COM D25 and MM1). The change in phosphorylation status of T308 was also investigated and found to be effected by VEGF. This effect was very cell type dependent. Our findings indicate that migration, in response to the addition of exogenous $VEGF_{121}$, is only upregulated in cell lines originating from dysplastic lesions and tumours. Cancer-associated fibroblasts were also stimulated to migrate. By contrast, however, $VEGF_{121}$ did not stimulate HaCaT and normal oral mucosal fibroblast cell migration (Fig. 2 and 3). *Ex vivo* IHC studies indicated that both of the phosphorylated motifs of Akt were overexpressed in tumour patients compared to normal patients (Fig. 4).

Our migration data is in agreement with previously published studies, where tumour-associated cells such as endothelial cells [22] and monocytes [23] were stimulated to migrate in response to VEGF. However, our data for the epithelial cell line, HaCaT, is contrary to data from Yang et al. (2009) who demonstrated that $VEGF_{165}$ enhanced HaCaT cell migration [24]. Response of cancer cells to VEGF in terms of Akt activation is summarised in Fig. 5. It should be noted that this is an over simplified view of events. Two cell lines, TR146 and COM D25, were stimulated to migrate in response to VEGF. These cell lines also displayed an increase in phosphorylation of Akt at S473 and T308 in response to VEGF. The addition of the inhibitor PI103 caused a decrease in the migration of these cell lines into 3D collagen gels.

Tumour progression requires both positive and reciprocal feedback between CAF and cancer cells. Initially, this is manifest by a change in

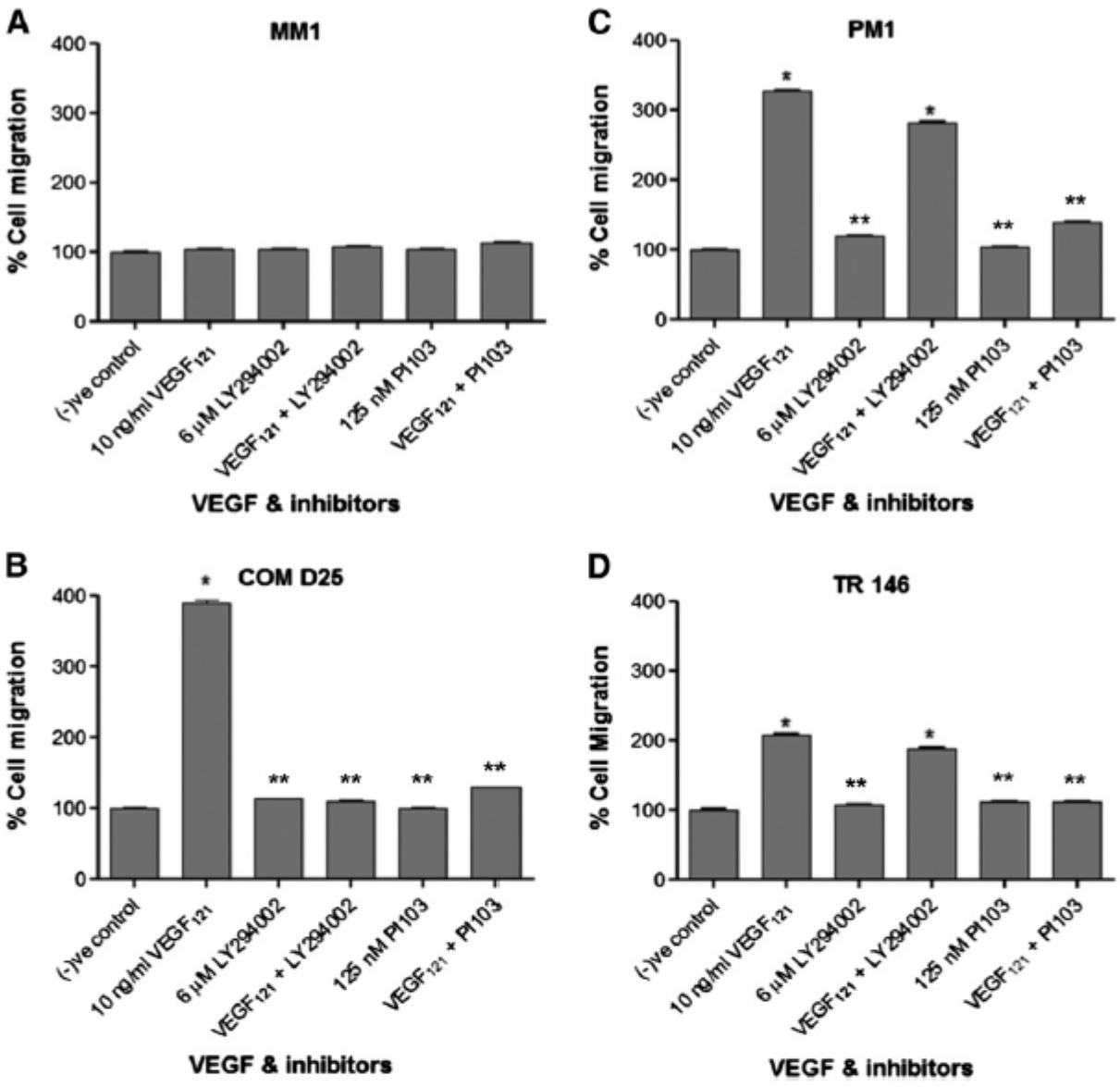

Fig. 3. VEGF stimulated cells from a dysplastic lesion and oral cancer cells to migrate in the collagen gel assay compared with the negative control: (A) Normal oral mucosal fibroblasts (MM1) were not stimulated to migrate into the collagen gel in response to VEGF and the inhibitors (baseline, $n = 30$) ($p > 0.05$). (B) Mouth cancer-associated fibroblasts (COM D25) were stimulated to migrate ($n = 33$) significantly by VEGF (baseline, $n = 8$) ($p < 0.05$). There was no effect of LY294002 and PI103 alone ($n = 9$ & 8, respectively) or in combination with VEGF ($n = 9$ & 10, respectively) on COM D25 cell migration. (C) PM1 were stimulated to migrate by VEGF alone ($n = 7$) and in combination with LY294002 ($n = 6$) ($p < 0.05$) (baseline, $n = 2$), and no effect with LY294002 & PI103. (D) TR146 were also stimulated to migrate by VEGF alone ($n = 15$) and in combination with LY294002 ($n = 13$) ($p < 0.05$) (baseline, $n = 7$), and LY294002 and PI103 had no effect. $n =$ number of cells migrated. Asterisk (*) indicates the significant changes compared with negative control. Double asterisk (**) indicates the significant changes compared with VEGF.

phenotype of normal fibroblasts to that of CAF, which occurs in response to various growth factors, including VEGF, which are secreted by the tumour cells [25]. However, it may be that these stromal cells themselves are predisposed to respond in a certain way to these growth factors and normal fibroblasts are not [26]. The CAF act as a source of various types of protease activity [27,28] allowing the cells to play a role in the invasive and metastatic process by remodelling the extracellular matrix. The CAF may also stimulate epithelial to mesenchymal transition of tumour cells through secretion of various growth factors or the response to breakdown products of the extracellular matrix [28,29]. The data presented here is the first evidence that oral cancer-associated fibroblasts are stimulated to migrate by exogenous VEGF treatment.

Experiments using different migration assays and the small molecule PI3K-Akt pathway inhibitors, LY294002 and PI103, show that treatment of cancer-associated fibroblasts, cells from a dysplastic lesion and cancer cells with these inhibitors alone is sufficient to inhibit VEGF-induced migration. PI103 appears to be a better inhibitor of cellular migration and phosphorylation of Akt. This may be due to the fact that PI103 can act at multiple places on the PI3K-Akt pathway. PI103 also blocks mTORC2 from phosphorylating Akt at S473 and mTORC1 further downstream [30]. This suggests that PI103 has three chances of blocking activity on the one pathway. However, the effects seen here could be PI103 affecting other pathways. In the long term, when considering PI103 as a possibly therapeutic agent, the multi-target effects may be fortuitous and ultimately represent an efficacious strategy for blocking metastases.

Our data support a model where the stimulation of PI3K-Akt activity by VEGF is a mechanism that drives cell migration in CAF and cancerous cells. Akt has been shown to be critically involved in VEGF-induced endothelial cell migration [31]. Full Akt kinase activity is dependent upon phosphorylation at residues T308 and S473 and this is greatly increased by growth factor receptor signalling [32]. Regardless of the activation mechanism, once phosphorylated, Akt loses its PIP3 binding requirement and translocates to distinct subcellular compartments, including the nucleus, mitochondria and other organelles [33]. Akt then transduces the signal by phosphorylating numerous substrate proteins, including both cytoplasmic and nuclear proteins. Accordingly, it is not unexpected that Akt activity can be found in both the cytoplasm and nucleus [34]. Although it has been suggested that differential phosphorylation of T308 and S473 may modulate the substrate selectivity of Akt, a clear picture of this is yet to emerge [32].

We have found higher VEGF expression in HNSCC patient samples compared to dysplastic patients. In agreement with this finding, it has been demonstrated previously that normal or mildly dysplastic oral

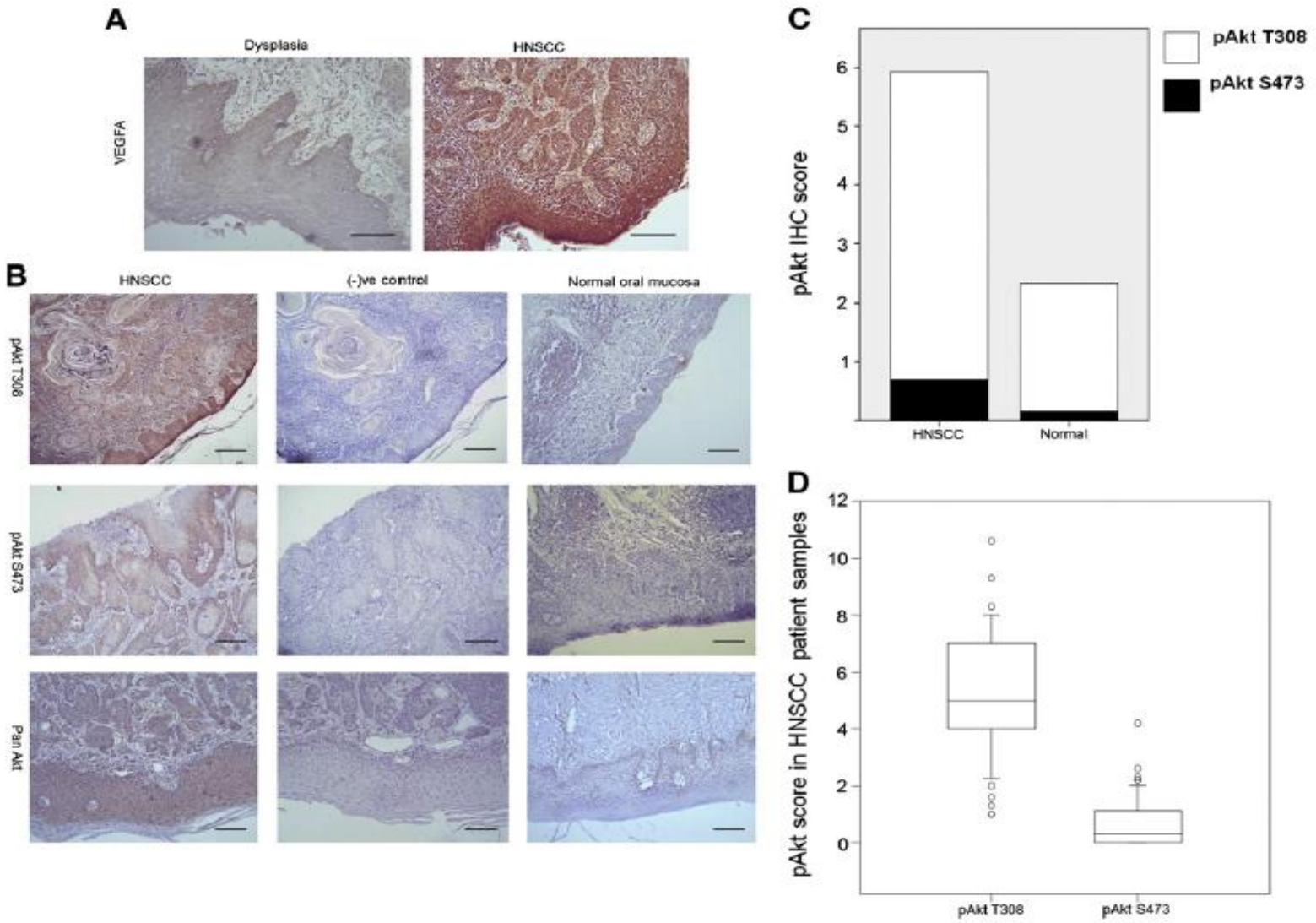

Fig. 4. Immunohistochemistry (A) showed significantly greater staining of VEGF A in the HNSCC tissues compared to dysplastic tissues ($p = 0.0001$). (B) VEGF positive carcinoma tissues were then stained for pAkt. Blocking peptides were used to test the efficacy of the antibodies and were tested on duplicate sections of those that had been highly stained for pAkt S473 and pAkt T308 using the antibodies alone. These were used as negative controls. Normal tissues were also used as negative controls, where no staining was observed. All the representative images were taken at ×100 magnification except for those stained for VEGF, which were taken at ×200. (C) pAkt S473 and pAkt T308 showed more intense staining in HNSCC tissues compared to normal tissues (Mann Whitney *U* test, $p < 0.05$). (D) Phosphorylation of Akt at residue T308 was found to be significantly higher in HNSCC patient samples compared to phosphorylation at S473 (Mann Whitney *U* test, $p < 0.001$). Scale bar = 5 mm.

epithelia did not exhibit VEGF expression or that the expression was significantly lower than in neoplastic epithelia [35–37]. VEGF expression was also upregulated in cancerous tissues compared to normal oral mucosa [38]. In this study we have also found nuclear localisation of pAkt T308 in VEGF positive oral carcinoma tissues, whereas pAkt S473 was mostly diffuse or localised in the cytoplasm. The Ringel group showed that the localisation of activated Akt differs between the two forms of thyroid cancer, but nuclear localisation is associated with tumour invasion in both subtypes [39]. Although Akt has been reported to be rich in the nucleus in many cancer cells, the mechanism of translocation, biological importance and activity have not yet been confirmed [40].

Even though it is not proven that the phosphorylation of Akt at T308 alone is sufficient for the progression of cancer, studies with human non-small cell lung cancer reported that pAkt T308 is a more reliable biomarker for the protein kinase activity of Akt in tumour samples than S473 [41]. We report here that phosphorylation of Akt at T308 is higher in the invasive oral carcinoma patient samples. It will therefore be beneficial to further investigate the role of pAkt T308 in VEGF-positive oral cancer progression and its downstream signalling pathways.

5. Conclusion

Studies into the role of Akt in VEGF-induced cancer cell migration have produced potentially conflicting results, which reveal both positive and negative effects of the inhibitors. This paradox could be, in part, explained by experimental design *i.e.* the migration assay format and treatment period employed. In addition, intra-tumoural variation reflective of the existence of multiple sub-clonal tumour populations may also contribute. This variation might correspond to an essential, yet unrecognised, determinant for the appearance of secondary drug resistance [42]. Inconsistent responses to targeted therapies illustrate the requisite for personalised cancer treatment, where the importance of recognising and appreciating the specific intricacy and variability of a tumour is paramount. The data presented exemplify that to enable the design of efficacious chemotherapeutic regimes, there is an absolute requirement for tumours to be precisely characterised.

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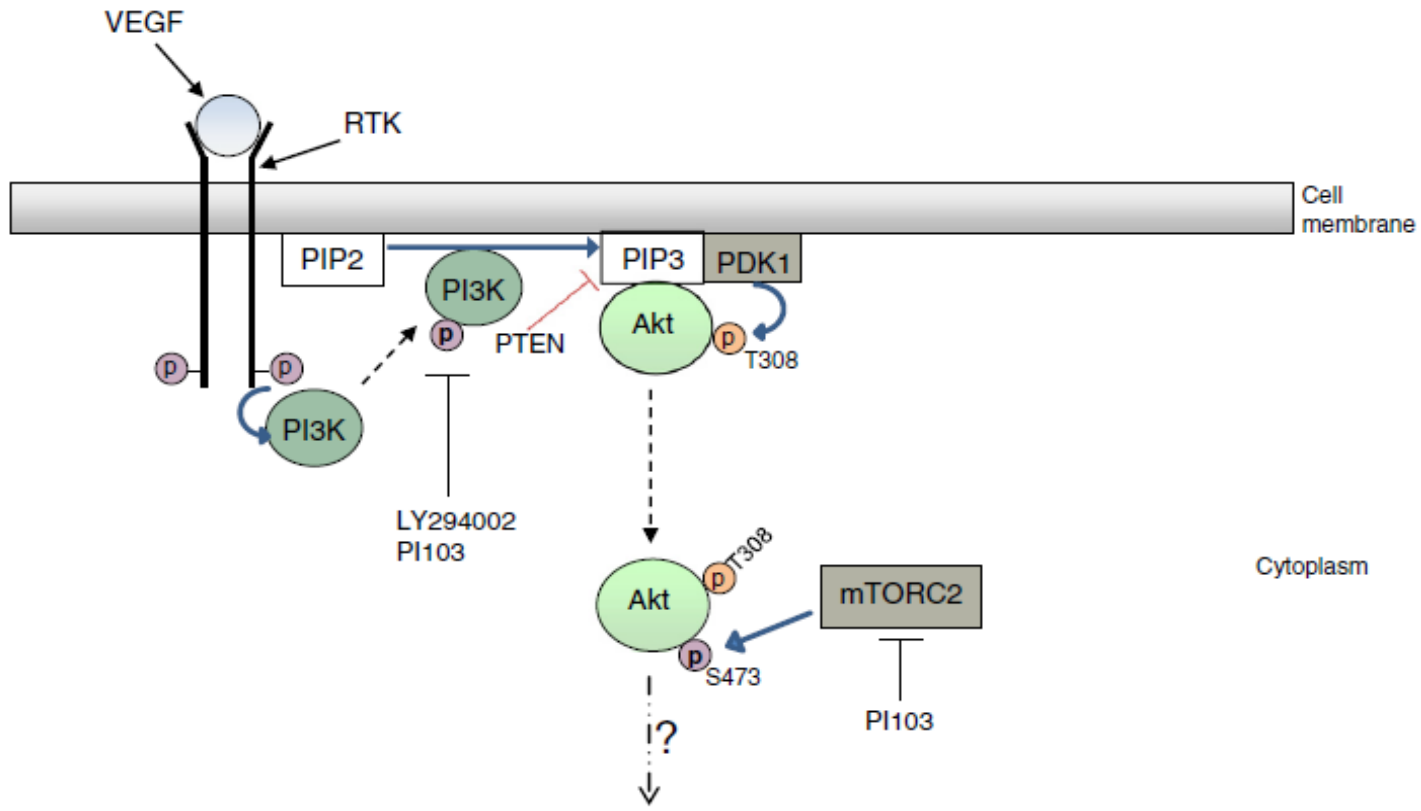

Blue arrow-Phosphorylation, Red block head- Dephosphorylation, Dashed arrow- Localisation, Black block head- Inhibition

Fig. 5. Proposed PI3K-Akt signal transduction pathway in oral cancer cell migration. RTK—receptor tyrosine kinase, PIP2—phosphoinositide 4,5-bisphosphate, PIP3—phosphoinositide 3,4,5-triphosphate, PI3K—phosphoinositide 3-kinase, PTEN—phosphatase and tensin homologue, PDK1—phosphoinositide-dependent kinase-1, mTORC2—mammalian target of rapamycin complex-2.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2014.02.004.

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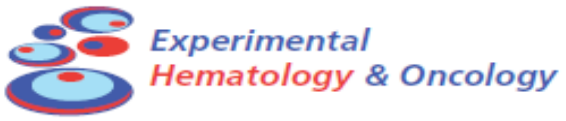

RESEARCH Open Access

Activation of Akt at T308 and S473 in alcohol, tobacco and HPV-induced HNSCC: is there evidence to support a prognostic or diagnostic role?

Mohammad R Islam[1], Ian R Ellis[1], Michaelina Macluskey[1], Lynda Cochrane[2] and Sarah J Jones[1*]

Abstract

Background: Tobacco, alcohol and HPV infection are associated with increased risk of HNSCC. However, little is known about the underlying signaling events influencing risk. We aimed to investigate the relationship between these risk factors and Akt phosphorylation, to determine prognostic value.

Method: VEGF-positive HNSCC biopsies, with known HPV status, were analyzed by immunohistochemistry (IHC) for Akt, phosphorylated at residues S473 and T308. Comparisons between the tissues were carried out using a Mann–Whitney *U* test. Associations between the variables and continuous immunohistochemical parameters were evaluated with general linear models. Patient characteristics and pAkt IHC score were analyzed for possible association with overall survival by Cox proportional hazard models.

Results: Immunohistochemistry revealed that cancer patients had significantly higher levels of pAkt T308 than S473 ($P < 0.001$). Smoking and alcohol were found to be independent risk factors for Akt phosphorylation at T308 ($P = 0.022$ and 0.027, respectively). Patients with tumors positive for HPV or pAkt S473 had a poorer prognosis ($P = 0.005$, and 0.004, respectively). Patients who were heavy drinkers were 49 times more likely to die than non-drinkers ($P = 0.003$). Patients with low pAkt T308 were more likely to be HPV positive ($P = 0.028$). Non-drinkers were also found to have lower levels of pAkt T308 and were more likely to have tumors positive for HPV than heavy drinkers ($P = 0.044$ and 0.007, respectively).

Conclusion: This study suggests different mechanisms of carcinogenesis are initiated by smoking, alcohol and HPV. Our data propose higher phosphorylation of Akt at T308 as a reliable biomarker for smoking and alcohol induced HNSCC progression and higher phosphorylation of Akt at S473 as a prognostic factor for HNSCC.

Keywords: pAkt T308, pAkt S473, HNSCC, Risk factors, Prognosis, Biomarker

Background

HNSCC (Head and Neck Squamous Cell Carcinoma) includes cancers that involve the oral cavity, pharynx and larynx. Each year there are approximately 400 000 cases of cancer of the oral cavity and pharynx, with 160 000 cancers of the larynx, resulting in approximately 300 000 deaths [1]. It is the sixth most common type of cancer worldwide with a five year survival rate of 40-50%, which has shown only moderate improvement over the last two decades [2]. Two modifiable risk factors, tobacco use and alcohol consumption, are thought to explain approximately 75% of the incidence of HNSCC (up to 100 times higher for both) [3]; another important risk factor is HPV infection, which has been detected in around 20% of all cases [4].

Recent studies have focused on the genetic and epigenetic alterations of HNSCC, providing a better understanding of the molecular events underlying the pathogenesis of HNSCC [5,6]. One of the most frequently altered signaling pathways in HNSCC is the PI3K/Akt cascade [6]. Akt, also known as PKB, is a serine-threonine protein kinase and is

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central to the phosphatidylinositol 3-kinase (PI3K) signaling pathway [7-9]. PI3K is activated by tyrosine-kinase transmembrane receptors and other signaling intermediates, such as Ras oncogenes and G proteins [10]. PI3K then phosphorylates PtdIns (4,5) P2 (PIP2) yielding PtdIns (3,4,5) P3 (PIP3), which serves as an anchor for intracellular proteins (primarily mediated by pleckstrin homology domains), including Akt amongst others. Membrane-bound Akt is phosphorylated at T308 in the catalytic domain by the kinase PDK1 and at S473 in the regulatory domain by mTORC2 [11,12]. Full Akt kinase activity is dependent upon phosphorylation at both T308 and S473 residues and this is greatly increased by growth factor receptor signaling [13]. PIP3 is converted back into PIP2 through the action of the lipid phosphatase PTEN, thus terminating the PI3K-initiated signal and avoiding further Akt activation [14]. Growth factor receptor over-expression [15,16], mutation and down-regulation of PTEN protein [17] and amplification of the PIK3CA gene (the gene coding for the catalytic unit of PI3K) [18] can lead to increased Akt activity. Enhanced Akt activity has indeed been found in 20 to 60% of tumor samples and in the majority of HNSCC-derived cell lines [18-21]. Once Akt is phosphorylated and activated, it is capable of phosphorylating multiple substrates generating diverse cellular processes, such as metabolism, proliferation, survival and protein synthesis [22].

An increasing number of mucosal changes and cellular atypia occur over large areas of the carcinogen-exposed upper aero-digestive tract epithelium, which initiate the stepwise carcinogenesis process in HNSCC. Acquisition of a transformed phenotype and accumulation of specific molecular genetic events are associated with this process [23-28]; yet histopathological evaluation remains the time honored method in risk assessment of carcinoma lesions. In a search for better biological models of risk, Akt activation was recently identified as an early cellular response to carcinogen exposure and may be a significant step in environmental carcinogenesis [29]. Akt activation has also been found to correlate with squamous cell carcinoma progression from normal epithelium to invasive cancer [30].

These observations encouraged us to investigate the role of demographic, pathological and major risk factors (smoking, alcohol and HPV) of HNSCC patients on the activation of Akt (phosphorylation of Akt at Threonine 308 and Serine 473) and to determine their prognostic role.

Results

Analysis of patient details

58 HNSCC patient details were analyzed in this study. 33 (57%) were male and 25 (43%) female with ages ranging from 36 to 97 years (median age 64 years). 42 (72%) were smokers and 16 (28%) non-smokers. 7 patients (12%) were non-drinkers, 21 (36%) medium or moderate drinkers and 30 (52%) were heavy drinkers. 31 (53%) patients had negative and 27 (47%) had positive nodal metastasis. Half of the cohort was HPV positive. 43 (74%) patients had T1/T2 and 15 (26%) had T3/T4 tumor size with 5 (9%) of them grade I, 39 (67%) of them grade II and 14 (24%) of them grade III. These data are summarized in Table 1.

Immunohistochemistry for Akt phosphorylation

Both normal and VEGF-positive carcinoma patient samples were stained with pAkt S473 and pAkt T308 antibodies. Some samples which were highly stained for pAkt S473 and pAkt T308 were then selected and tested with the blocking peptide for the respective antibody and were used as negative controls (Figure 1A). No staining was observed in the blocking peptide treated tissues and this confirmed the specific binding of the antibodies.

Normal tissue samples were also regarded as negative controls and the level of Akt phosphorylation at T308 was higher in the HNSCC group than the control (median 5.8 vs 2.0, $P < 0.001$) (Figure 1B). Normal tissue adjacent to the tumors was also tested and the resultant data indicated that there was very low or no pAkt T308/pAkt S473 present (Figure 1C). There is also some evidence to suggest higher levels of pAkt S473 in the cancer group than the controls ($P = 0.054$) (Figure 1B). There is a statistically significant difference between pAkt T308 and pAkt S473 levels in the cancer patients (pAkt T308, median 5.8 vs pAkt S473, median 0.3, $P < 0.001$) (Figure 1B). VEGFA is not correlated with pAkt T308 ($r = 0.062$, $P = 0.644$) and pAkt S473 ($r = 0.181$, $P = 0.175$). Table 1 compares the characteristics between pAkt expression groups. All the samples were found to be phosphorylated at Akt T308 so there is no 'no phosphorylation' group for this residue. On the other hand, there is no 'high phosphorylation' group for pAkt S473.

Association of patient characteristics with Akt phosphorylation

Smoking and alcohol were found to be independent risk factors for phosphorylation of Akt at T308 ($P = 0.022$ and 0.027, respectively) but not for pAkt S473 ($P = 0.449$ and 0.968, respectively). HNSCC with nodal metastasis was associated with a higher level of pAkt T308 than HNSCC without nodal metastasis ($P = 0.018$). Smokers and HNSCC with nodal metastasis were found to have higher levels of Akt phosphorylated at T308 than non-smokers ($P = 0.022$) and HNSCC without modal metastasis ($P = 0.018$) patients, respectively. HPV-negative patients exhibited higher levels of pAkt T308 compared to those who were HPV positive ($P = 0.028$). There is some evidence to

Table 1 Demographic, behavioral and pathological data by pAkt status

		Akt T308 phosphorylation status				Akt S473 phosphorylation status			
	n	High (n = 40) %	Medium (n = 8) %	Low (n = 10) %	p	Medium (n = 5) %	Low (n = 30) %	None (n = 23) %	p
Gender					0.109				0.737
Male	33	62	63	30		60	50	65	
Female	25	38	37	70		40	50	35	
Age					0.233				0.376
<65 years	30	50	50	60		80	43	57	
≥65 years	28	50	50	40		20	57	43	
Location					0.217				0.094
FOM	10	15	25	20		20	17	17	
RMT	8	10	13	30		0	10	22	
SP	5	10	0	10		40	10	0	
Tong	27	53	50	20		20	47	53	
Alv	4	5	0	20		0	10	4	
Other	4	7	12	0		20	6	4	
Tumor size					0.173				0.213
T1-T2	43	70	25	90		60	73	78	
T3-T4	15	30	75	10		40	27	22	
Grade					0.523				0.788
I	5	8	12	10		0	7	13	
II	39	65	75	70		80	66	65	
III	14	27	13	20		20	27	22	
Lymph node metastasis					0.018				0.327
Positive	27	58	12	30		40	50	43	
Negative	31	42	88	70		60	50	57	
HPV status					0.028				0.301
Positive	29	40	63	80		40	43	61	
Negative	29	60	37	20		60	57	39	
Smoking					0.022				0.449
Yes	42	82	50	50		80	70	74	
No	16	18	50	50		20	30	26	
Alcohol					0.027				0.968
Non-drinker	7	10	0	30		0	17	9	
Medium drinker	21	27	62	40		40	33	39	
Heavy drinker	30	63	38	30		60	50	52	

Note: The General linear model was used for hypothesis testing of the relationship between different variables and pAkt. The P value was obtained from univariable analysis. Percentages represent the column percentages within variable so that the balance between the pAkt groups could be assessed.
Abbreviations: *FOM* Floor of the mouth, *RMT* Retromolar trigone, *SP* Soft palate, *Tong* Tongue, *Alv* Alveolus.

suggest that heavy drinking patients had higher levels of pAkt T308 than medium drinkers (P = 0.063).

Adjusted by four independent variables, smoking, drinking, nodal status and age, the general linear model accounts for 65.2% ($R^2 = 0.652$) of the total variation in pAkt T308 levels (Table 2). This model's significance statistics for the F-statistic (P < 0.001) indicate that there is only a very small chance that the observed correlation between one or more of the independent variables and the dependent variable is due solely to random sampling error.

Smoking (P = 0.027), drinking habit (P < 0.001) and age (P = 0.031) showed a significant effect on the phosphorylation of Akt at T308. Alcohol and age, alcohol and nodal involvement, smoking and nodal involvement and

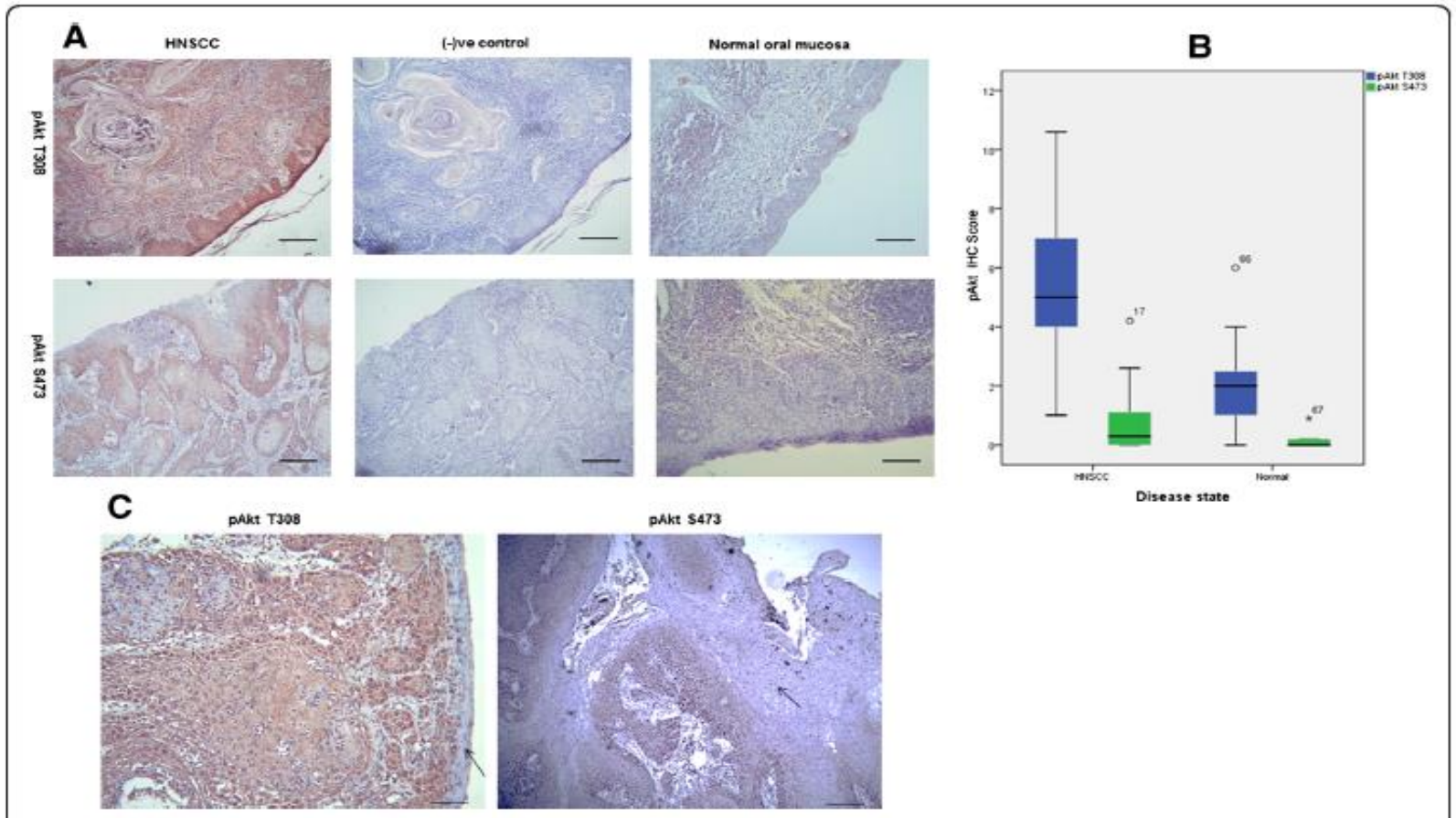

Figure 1 VEGF positive carcinoma tissues were stained with pAkt antibodies. Blocking peptides were used to test the efficacy of the antibodies and were tested on duplicate sections of those that had been highly stained for pAkt S473 and pAkt T308 using the antibodies alone. These were used as negative controls. Normal tissues were also used as negative controls, where no staining was observed. All the representative images were taken at x200 magnification except for those stained for pAkt T308, which were taken at x100. **(A)** pAkt S473 and pAkt T308 showed more intense staining in HNSCC tissues compared to normal tissues ($P < 0.05$). **(B)** Phosphorylation of Akt at residue T308 was found to be significantly higher in HNSCC patient samples compared to phosphorylation at S473 ($P < 0.001$). **(C)** Phosphorylation status of Akt in non-tumor part of cancer tissues. Very weak or no phosphorylation of Akt at both T308 and S473 was observed in the non-tumor part of HNSCC tissues. The arrow indicates the non-tumor area of the tissues. Images were captured at x200 magnification.

smoking and alcohol are strongly correlated with the phosphorylation of Akt at T308 in pairwise combination ($P = 0.009$, 0.038, 0.049 and 0.052, respectively). Moreover, smoking, nodal involvement, age and alcohol, nodal involvement, age also have a strong correlation with the phosphorylation of Akt T308 ($P = 0.019$ and 0.022, respectively). None of these independent variables correlated with the phosphorylation of Akt at S473 (univariate and multivariate, $P > 0.05$).

Non-drinking patients had lower levels of Akt phosphorylated at T308 and were more likely to be infected by HPV than heavy drinkers (χ^2 $P = 0.044$ and 0.007, respectively). Also, HPV infected patients were shown to have lower levels of Akt phosphorylated at T308 than the non-infected patients (χ^2 $P = 0.028$).

Survival Analysis

Levels of pAkt S473 and pAkt T308, as determined by IHC, were examined for association with overall survival using Cox's proportional hazard model (Table 3). In multivariate analysis, pAkt S473 level, tumor size, alcohol consumption, age and patients' HPV status had significant effects on overall survival ($P = 0.005$, 0.005, 0.021, 0.007 and 0.004, respectively).

The rate of deaths in patients with medium levels of phosphorylated Akt S473 was 438 times higher than in those with none and 21.5 times higher than in those with low levels ($P = 0.001$ and $P = 0.036$, respectively, Table 3). Patients with tumors of size T3/T4 died more rapidly than patients with tumors T1/T2 ($HR = 15.2$, $P = 0.005$). Death rates in heavy drinkers were 49.4 times higher than those who consumed no alcohol ($P = 0.006$). Older patients (65 years of age or over) were 46.8 times more likely to die than those of 65 years of age ($P = 0.007$). Finally, HPV positive patients were 89.4 times more likely to die than those with a negative status ($P = 0.004$).

Discussion

To our knowledge this is the first report to show the relationships between the major risk factors for HNSCC

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Table 2 General Linear Model (Multivariate analysis)

Independent variables	F	P
Corrected model	4.06	<0.001
Smoking	5.30	0.027
Alcohol	10.56	<0.001
Nodal status	1.31	0.260
Age	4.98	0.031
Smoking * Alcohol	3.20	0.052
Smoking * Nodal status	4.12	0.049
Smoking * Age	0.01	0.919
Alcohol * Nodal status	3.56	0.038
Alcohol * Age	5.31	0.009
Nodal status * Age	2.80	0.102
Smoking * Alcohol * Nodal status	0.17	0.682
Smoking * Alcohol * Age	0.08	0.774
Smoking * Nodal status * Age	6.00	0.019
Alcohol * Nodal status * Age	5.73	0.022
$R^2 = 0.652$		

Note: After adjusting Smoking, Alcohol, Nodal Status and Age, this model accounts for 65.2% of the total variations in pAkt T308 level. R^2 = Coefficient of determination, F = F-statistics.
Dependent variable: pAkt T308 score.

(alcohol, smoking and HPV) and Akt activation (both at residues T308 and S473) at the protein level using an immunohistochemical staining method (IHC), in surgically resected specimens. Most studies to date have used IHC to assess the prognostic value of Akt activation in HNSCC, but have focused only on phosphorylation of residue S473. As the differential phosphorylation of Akt at the two sites may modulate downstream substrate selectivity and subsequent bioactivity [13], it is not surprising that Akt phosphorylated at any single site could perform certain cellular bioactivities. Two different mechanisms are involved in phosphorylating Akt, therefore overexpression or amplification of any components in these mechanisms may result in over-phosphorylation at any one of the two sites. It is therefore worth studying the phosphorylation status of Akt at both sites in HNSCC specimens to elucidate their different roles.

As only VEGFA positive HNSCC biopsy samples were selected in this study, no statistically significant correlation was found between VEGF and pAkt. The present study showed that Akt was significantly phosphorylated at T308 in VEGF positive HNSCC rather than S473. Alcohol and smoking were positively correlated with pAkt T308 activation but not with pAkt S473. Moreover, Akt activated at T308 showed a significant relationship with lymph node metastasis, which suggests that pAkt T308 may be concerned with invasion and metastasis. This data is similar to our *in vitro* data concerning the migration of tumor cells in response to VEGF, which suggests

Table 3 COX proportional hazard model- time to death

		Unadjusted HR			Adjusted HR		
		HR	95% CI	P	HR	95% CI	P
pAkt S473	Overall			0.168			0.005
	Med:No	6.27	0.87, 45.3	0.069	438	10.9, 1755	0.001
	Med:Low	1.83	0.38, 8.88	0.456	21.5	1.23, 376	0.036
Tumor size	T3/T4:T1/T2	4.59	1.39, 15.2	0.013	15.2	2.28, 102	0.005
Alcohol	Overall			0.005			0.021
	Heavy:Non-drinker	15.4	2.74, 86.7	0.002	49.4	3.04, 801	0.006
	Heavy:Moderate	3.19	0.62, 16.5	0.166	2.69	0.35, 20.8	0.343
Age	≥65:<65	5.41	1.17, 25.05	0.031	46.8	2.81, 781	0.007
HPV	+ve:−ve				89.4	4.05, 1973	0.004
Gender	F:M	1.40	0.42, 4.63	0.581			
Tumor size	Overall			0.310			
Nodal status	+ve: −ve	1.49	0.43, 5.10	0.529			
Smoking	No:Yes	1.45	0.42, 4.95	0.556			
pAkt T308	Overall			0.984			
	Low:High	1.02	0.21, 4.92	0.985			
	Med:High	1.16	0.24, 5.63	0.858			

Note: Unadjusted HR obtained from univariable analysis and adjusted HR from multivariable analysis after adjusting tumor size, alcohol, age, HPV and pAkt S473. All the variables are categorical and HR = exp (B). Abbreviations: *HR* Hazard ratio, 95% *CI* 95% confidence interval.

that migration of oral adeno-squamous cancer cells is dependent on Akt T308 phosphorylation. Our study also disclosed that Akt phosphorylated at both residues controls oral cancer cell motility [31], but it should be remembered that studies performed with cultured cells or tissue models may produce different results. *In vivo*, tumor progression requires both positive and reciprocal feedback between the components of the tissue microenvironment and cancer cells [32].

The activation of Akt in response to alcohol exposure is an important contributor to the molecular effects of excessive alcohol consumption [33]. In 2003, West showed that redundant Akt activation by nicotine and nicotine-derived nitrosamine ketone (NNK) could contribute to tobacco-related carcinogenesis [29]. A study by the Gonzalez group in 2005, revealed that Akt activation was correlated with concomitant PI3K accumulation and PTEN down-regulation in HNSCC, reflecting an early biochemical effect in response to nicotine [18]. Combined with these data, our study supports the basic hypothesis that Akt activation (especially at T308) is a key step in the progression of HNSCC caused by alcohol and smoking. HPV infection, another risk factor for HNSCC, was found to be negatively correlated with Akt activation at T308, as HPV positive HNSCC patients showed lower levels of pAkt T308. Non-drinking patients had lower levels of activated Akt at T308 too and there were more HPV positive patients among non-drinkers than amongst the heavy drinkers. Earlier epidemiologic research supports this data, that is non-smokers and light or non-drinkers are more likely to have tumors positive for HPV than are heavy smokers and drinkers [34]. Molinolo *et al.* [35] showed in their study that HPV positive HNSCC patients over-activate Akt at S473 and mTOR. Although we have not found any association between HPV infection and pAkt S473 activation, this may suggest that there are two different mechanisms of cancer progression initiated by alcohol, smoking and HPV. The Kelsey group study (2007), strongly supports the emerging view that the etiology of HPV related HNSCC is distinct from that of HNSCC tumors associated with smoking and drinking [36]. Increased Akt activation at T308 by excessive alcohol and smoking may be responsible for cancer development and progression, including metastasis, whereas HNSCC by HPV infection may over-activate Akt at S473 and be responsible for poor survival.

In this study we show that increased pAkt S473 levels in HNSCC are a strong predictor for poor patient outcome. In the multivariate Cox proportional hazard model, adjusted for well recognized prognostic indicators (e.g. tumor size and age), pAkt S473 status remained a strong predictor. This is corroborated by three other studies which have shown that pAkt activated at S473 is associated with poor prognosis in oral cancer [37-39]. Although further molecular analysis is needed to investigate the mechanism of smoking and alcohol related HNSCC development, we can propose pAkt T308 as a reliable biomarker for smoking and alcohol induced HNSCC progression.

Conclusions

The predictive role of Akt activation in HNSCC suggests that targeting PI3K/Akt and mTORC2/Akt pathway along with RTK (receptor tyrosine kinase) might be a useful strategy for therapy in this disease. A large cohort with a longer follow-up of pre-neoplastic and HNSCC lesions is needed to more accurately define the role of Akt activation in carcinogenesis and to integrate this data into a risk model for carcinoma development and progression. In conclusion, our findings suggest that targeting Akt activation might be of interest as part of a combination therapy in HNSCC, as described earlier [40].

Methods

Patients

Ethical approval (ID: LEC271/03) was granted for the prospective collection of tissues which were stored at the Tayside Tissue Bank. In total 64 HNSCC and 11 normal oral mucosal tissues (from non-tumor patients) were collected from patients treated at Ninewells Hospital Tayside. Baseline data obtained from patient charts included age, sex, histology, site, drinking and smoking status, nodal involvement, survival and follow-up data. Patients were followed-up for a total of 66 months (median, 40 months) after diagnosis.

Some continuous variables (such as age, drinking and smoking status) were changed into categorical variables in this study with clear justification (statistical and/or clinical reasons). Age was grouped as '<65 years of age' and '≥65 years' because approximately 50% patients were above/below 65 and a growing number of patients with Head and Neck Squamous Cell Carcinoma (HNSCC) are aged 65 and older [41]. Patients who consumed alcohol were referred to as 'drinkers' throughout this article. Drinkers were categorized as non-drinkers, medium or moderate drinkers (less than 7 units per week for women and 14 units per week for men or occasional or social, regarded as low risk group by NIAAA), and heavy drinkers (over 7 units per week for women and 14 units per week for men) according to the National Institute of Alcohol Abuse and Alcoholism [42]. Smoking status was classified as non- or light smokers (less than 5 cigarettes per day) and smokers (more than 5 cigarettes per day) after reviewing the literature [43-45].

HNSCC tissues were stained for VEGFA expression by IHC [31] and tissues with IHC scores of more than 3 were selected and regarded as positive. Tissues were also analyzed for HPV DNA by PCR, automated DNA sequencing and the SPF_{10}-$LiPA_{25}$ method [46].

Immunohistochemistry

The paraffin-embedded tissues were cut into 5 μm sections, dewaxed in xylene and then rehydrated in serial ethanol solutions, before washing in distilled water for 5 minutes. 58 VEGFA positive HNSCC and 11 normal mucosal samples were then probed with pAkt T308 (#2965) and pAkt S473 (#4060) antibodies according to the manufacturer's instructions (Cell Signaling Technology Inc., Danvers, MA, USA). In brief, after the deparaffinization and rehydration process, antigens were unmasked by boiling in 10mM Sodium citrate buffer (pH 6.0) using a microwave, followed by maintenance at a sub-boiling temperature for 10 minutes and then cooling for 30 minutes on the bench top. 3% (v/v) H_2O_2 was then used as a peroxidase blocker and TBST (Tris buffered saline with 0.1% v/v Tween 20) for washing. Sections were then blocked with 5% (v/v) normal goat serum (NGS) plus TBST for 1 hour at room temperature. Sections were then incubated with antibodies against pAkt S473 (1:50) and pAkt T308 (1:50) diluted in 5% (v/v) NGS/TBST in a humidified chamber overnight at 4°C. After equilibration, sections were then washed three times with TBST and then incubated in signal stain boost detection reagent (HRP, rabbit #8114, Cell Signaling Technology) for 30 minutes at room temperature. Visualization was achieved by incubation with 3,3'-diaminobenzidine (DAB) (Sigma-Aldrich, MO, USA) for 5 minutes and counterstaining with Mayer's haematoxylin (Sigma) and eosin. Rehydration and mounting processes were then followed as described in the instruction manual (Cell Signaling Technology). Normal oral mucosal tissues were used as negative controls. The pAkt S473 and pAkt T308 antibodies were blocked using the respective blocking peptide (#1140 and 1145B, respectively, Cell Signaling Technology) by adding twice the volume of peptide as volume of antibody used, in a total volume of 100 μl. These tissues were also used as negative control.

IHC score

According to the scoring systems that have been reported previously in the literature, [47,48] with some modifications, pAkt staining scoring was performed as follows: stained sections were visualized using a light microscope at high power field and were evaluated by three observers without prior knowledge of the patients' characteristics. An intra-class correlation (inter-observer correlation) analysis using a mixed model and testing for consistency gave a Chronbach's alpha of more than 0.8. The cells showing cytoplasmic and/or nuclear staining were judged as positive. Five high power fields were selected randomly under the microscope. The average percentage of positive staining was calculated for each field. The average percentage of tissue staining was designated as 0 when less than 10% was stained, 1 when 10-25%, 2 when 25-50%, 3 when 50-75% and 4 when >75% of tissues stained. The intensity of tissue staining positively was categorized as follows: 0, no appreciable staining in tissues; 1, barely detectable staining as compared with stromal elements; 2, readily appreciable brown staining distinctly marking cell cytoplasm and/or nucleus; and 3, dark brown staining in tissues completely obscuring cytoplasm and/or nucleus. Scoring was performed according to the product of staining intensity and average percentage of tissue staining positively ranging from 0–12. In the following analysis, the level of Akt phosphorylation was evaluated using the pAkt index either as a continuous variable directly or categorized as no phosphorylation (IHC score 0), low phosphorylation (IHC score 0.1-2.0), medium phosphorylation (IHC score 2.1-5.0), high phosphorylation (IHC score 5.1-12.0) after reviewing a number of studies [37,49-60].

Statistics

Data were analyzed using the statistical package IBM SPSS 19.0. Comparisons between the tissues (HNSCC and normal) regarding the Akt phosphorylation were carried out using a Mann–Whitney *U* test. Associations between categorical demographic, pathological and behavioral factors were investigated using cross tabulation and Pearson chi-square test. Associations between these variables and continuous immunohistochemical parameters were evaluated with general linear models (both univariate and multivariate). Bonferroni's correction for multiple comparisons was applied where appropriate.

Patients' characteristics and pAkt IHC score were analyzed for possible association with overall survival by univariate and multivariate Cox proportional hazard models. Overall survival was defined as the time between diagnosis date and death or last follow-up date. Initially, explanatory factors were screened for univariate associations with death, using a method appropriate to the distribution of the data. If the two-sided P value was <0.300 for any variable it was considered as a candidate in multiple regression models (Hosmer-Lemeshow criterion). Variables with $P \geq 0.300$ were discarded at this stage. The assumption of proportional hazards was checked for independent variables by plotting the logarithm of the cumulative hazards functions. Starting with the set of variables identified for inclusion from the previous steps, a multiple Cox regression model was built using a step-wise approach.

All tests were two-sided, using the 5% significance level.

Abbreviations

HNSCC: Head and neck squamous cell carcinoma; pAkt T308: Akt phosphorylated at Threonine 308; pAkt S473: Akt phosphorylated at Serine 473; VEGF: Vascular endothelial growth factor; HPV: Human papilloma virus.

Competing interests

The authors declare that they have no competing interests.

Author contribution

MI participated in the design of the study, carried out the immunohistochemistry and drafted the manuscript. MI performed the statistical analyses with and under direction of LC. MM was granted ethical approval for collection and use of the biopsies and directed the associated VEGF and HPV studies. IE and SJJ conceived of the study, and participated in its design and coordination and helped to draft the manuscript. IE and SJJ were granted funding for the study and MI's studentship. All authors have read and approved the final manuscript.

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